REMARKS/ARGUMENTS

The Pending Claims

Claims 1-13 are pending and are directed to a compound of formula (I), methods of synthesizing the compound, and methods of using the compound to treat cancer in a human.

The Amendments to the Claims

Claim 10 has been amended to recite a method of synthesizing the compound of claim 1 comprising (a) coupling (1,1-Dialkyl-2-oxo-butylsulfanyl)-acetic acid or a specified derivative thereof to one of three specified compounds, and subjecting the product of the coupling reaction to an aldol condensation reaction. This amendment is supported by the specification at, e.g., paragraphs 0049, 0051, 0053, and 0054-0060. Accordingly, no new matter has been added by way of these amendments.

The Office Action

The Office Action rejects claims 1-13 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Claims 1-13 also are rejected under 35 U.S.C. § 112, first paragraph, for an alleged lack of written description and enablement. Reconsideration of these rejections is hereby requested.

Discussion of Indefiniteness Rejections

Claims 1-13 allegedly are indefinite under Section 112, second paragraph, because the term "hydrate" is not defined in the present application. Contrary to the assertion of the Office Action, the term "hydrate" is well-known in the art as a compound formed by the addition of water or its elements to a host molecule. Therefore, one of ordinary skill in the art of synthetic organic chemistry would understand what is meant by the term "hydrate."

Claim 10 is allegedly indefinite because the claim does not recite a reaction step for synthesizing the compound of claim 1. Claim 10 has been amended to recite specific reaction steps.

For the foregoing reasons, Applicants request withdrawal of the rejections under Section 112, second paragraph.

Discussion of Written Description Rejection

Claims 1-13 allegedly do not comply with the written description rejection under Section 112, first paragraph. The Office Action alleges that the present application does not adequately describe the term "hydrate." As discussed above, the term "hydrate" is well-known in the art as a compound formed by the addition of water or its elements to a host molecule. Furthermore, while different hydrates may exist for a particular compound (e.g., ½ hydrate, ¾ hydrate, etc.), one of ordinary skill in the art would be fully aware of the hydrates that are stoichiometrically feasible for a particular given compound.

Moreover, Applicants note that, in accordance with the present invention, hydrates are typically not actively synthesized but can occur during the production process or simply as a consequence of the hygroscopic nature of the initially anhydrous claimed compounds (see the specification at, e.g., page 5, lines 5-8, of paragraph 0024). In the pharmaceutical sciences it is also well established that the presence of a hydrate will not change the physico-chemical or pharmacological properties of a compound as compared to the non-hydrated form.

In view of the foregoing, claims 1-13 are adequately described in the present application, and the written description rejection under Section 112, first paragraph, should be withdrawn.

Discussion of Enablement Rejection

Claims 1-13 allegedly lack enablement under Section 112, first paragraph. Specifically, the Office Action alleges that the specification does not provide enablement for making solvates and hydrates of the claimed compounds. In addition, the Office Action contends that the present application provides insufficient guidance for a method of treating cancer using the claimed compound. This rejection is traversed for the reasons set forth below.

A. Enablement of Solvates and Hydrates

Contrary to the assertion of the Office Action, one of ordinary skill in the art would be able to prepare hydrates and solvates of the claimed compound based on the general knowledge in the art and the guidance provided by the specification. For example, Hofle, G.

& Reichenbach, H. (2005) published in *Anticancer Agents from Natural Products*, Cragg, G.M. et al., Eds., Taylor & Francis group, CRC Press, Boca Raton, FL, pp. 413-443 (submitted herewith) discloses epothilones as naturally occurring secondary metabolites in myxobacteria, and includes numerous epothilone synthesis routes that employ different solvates.

Furthermore, the thrust of the claimed invention is not the synthesis of hydrated or solvated compounds. Rather, such hydrates or solvates can occur during synthesis, for example, as a consequence of the hygroscopic nature of the initially anhydrous claimed compounds (see the specification at, e.g., page 5, lines 5-8, of paragraph 0024). The skilled artisan is well aware not only that a particular solvate or hydrate of a given compound may be required depending on the pharmaceutical dosage form to be employed, the route of administration, and/or the condition to be treated (e.g., for sustained release formulations), but also how to provide such solvate or hydrate.

B. Enablement of a Method of Treating Cancer

The pending claims are directed to 3-thia epothilones and methods of using such compounds to treat cancer. Applicants note that epothilones represent a new class of macrolide compounds naturally occurring in myxobacteria such as *Sorangium cellulosum*. The antitumor activity of epithilones is well established in the art; their cytotoxicity in cell cultures was reported immediately after their discovery in the late 1980s (see, e.g., Hofle, G. & Reichenbach, H., *supra*, page 421, lines 1-2). The particular mode of action exhibited by epothilones is based on the induction of tubulin polymerization and the stabilization of microtubules, which ultimately leads to programmed cell death (apoptosis).

Numerous preclinical studies have been performed since the early 1990s establishing that epothilones (both naturally occurring as well as synthetic and semisynthetic derivatives) act *in vivo*, that they inhibit human cancers, often resulting in cures of animals, and that they are superior to paclitaxel (e.g., Taxol™, Bristol-Myers Squibb) and other cytostatic drugs in many cases, especially with multidrug-resistant (MDR) tumors (see Hofle and Reichenbach, *supra*, page 438, lines 22-25).

In 1999, the first phase I clinical trials using epothilones were initiated. In 2003, phase II/III clinical trials employing epothilone B-lactam were performed (see Hofle and Reichenbach, *supra*, page 415, Fig. 21.2). Several epothilone derivatives have been clinically tested in patients suffering from a variety of carcinomas, including MDR cancers and tumors that are difficult to treat (see Hofle and Reichenbach at page 440, lines 40-42).

Thus, as early as the priority date of the present application, the anticancer activity of different epothilone derivatives was well known in the art. Moreover, the specification discloses that several epothilone derivatives have been clinically tested for the treatment of various cancers (see page 1, paragraph 0001). Indeed, the U.S. Food and Drug Administration very recently approved ixabepilone, a semi-synthetic analog of epothilone B, as the first epothilone-based medicament (IXEMPRA™, Bristol-Myers Squibb) for the treatment of certain chemotherapeutic-resistant breast cancers (see FDA Prescribing Information dated October 16, 2007, submitted herewith). The specification also discloses proliferation assays which demonstrate that the claimed compounds exhibit significantly improved effects on two well established tumor cell lines, namely MCF7 breast cancer cells and A549 lung cancer cells, as compared to paclitaxel (see page 24, paragraph 0063).

Based on the extensive amount of information available in the art concerning the antitumor activity of epothilones, and the *in vitro* data disclosed in the present application, there is no reason to question the *in vivo* efficacy of the claimed compounds. Other than generic assertions regarding the state of the art and the unpredictability of the claimed invention, which have been rebutted herein, the Office Action provides no specific reasons to doubt the enablement of claims 12 and 13. Accordingly, the enablement rejection under Section 112, first paragraph, is improper and should be withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned agent.

Respectfully submitted,

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Date: December 6, 2007

ANTICANCER AGENTS from NATURAL PRODUCTS

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Taylor & Francis
Taylor & Francis Group

Boca Raton London New York Singapore

A CRC title, part of the Taylor & Francis imprint, a member of the
Taylor & Francis Group, the academic division of T&F Informa plc.

Published in 2005 by
CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-1863-7 (Hardcover)
International Standard Book Number-13: 978-0-8493-1863-4 (Hardcover)
Library of Congress Card Number 2004065568

Library of Congress Cataloging-in-Publication Data

Kingston, David.
Anticancer agents from natural products / David Kingston, Gordon Cragg, David Newman.
p. cm.
ISBN 0-8493-1863-7
1. Antineoplastic agents. 2. Pharmacognosy. 3. Natural products. I. Cragg, Gordon M. L. II. Newman, David J. III. Title.

RS431.A64K545 2005
616.99'4061--dc22 2004065568

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21 Epothilone, a Myxobacterial Metabolite with Promising Antitumor Activity

Gerhard Höfle and Hans Reichenbach

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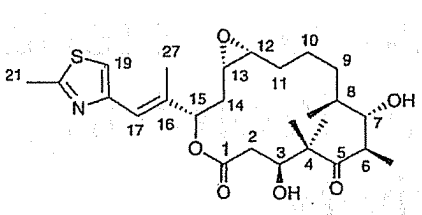
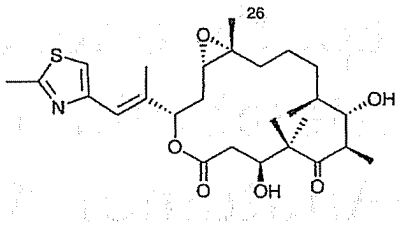
I. INTRODUCTION

Epothilone is a secondary metabolite of the myxobacterium *Sorangium cellulosum*. It is a macrolactone of a novel structural type characterized by an epoxy and a ketogroup in the lactone ring and a side chain with a thiazole ring (Figure 21.1) — functional groups from which the name epo-thi-lone was coined.[1] Two main variants, epothilone A and epothilone B, are normally isolated from the bacterial culture.[2,3] The B variant is a homolog with an extra methyl group at the epoxide, which makes it by a factor of 10 more active. Similarly, minor and trace components are always observed as pairs of homologs, with the B-series being invariably more active.[4] Epothilone, originally spelled epothilon, was discovered by us in ~~1986~~ because of its antifungal activity. Its structure was first disclosed in 1991 in the Annual Scientific Report of our institute, Gesellschaft für Biotechnologische Forschung (GBF).[1] The structure was elucidated mainly by spectroscopic methods and corroborated by x-ray crystallography, which also provided the absolute configuration.[3] Besides antifungal activity, strong cytotoxicity against mammalian cells was also reported. A German patent was filed in November 1991 (granted in May 1994), and an international one November 1992,[5] though it was withdrawn in April 1994 because the impressive antitumor activity of the epothilones was not, as yet, known, and there was limited interest in purely cytotoxic compounds. The patent strain, *Sorangium cellulosum* So ce90, was deposited at the German Culture Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ) in Braunschweig in October 1991 under number DSM 6773 and has been available to the public since then.



Epothilone A **1a**



Epothilone B **1b**

FIGURE 21.1 Structures of the major epothilones.

The discovery of epothilone was the result of a decade of basic research at the GBF with the aim of identifying new producers of diverse secondary metabolites among bacteria. This has indeed been achieved, with the discovery that myxobacteria synthesize a wealth of compounds, most of which possess diverse and novel structures and unusual and interesting mechanisms of action. Thus far, we have isolated and characterized more than 600 different compounds from those organisms, representing around 120 structural types,[6] and it is quite obvious that our many publications have stimulated more extensive investigation of myxobacteria for secondary metabolites.

In the 1980s and 1990s Taxol® (paclitaxel) was developed into a very successful antitumor drug with a new mechanism of action, viz, stimulation of tubulin polymerization and stabilization of microtubuli. As Taxol initially had to be produced from the bark of slow-growing Pacific yew trees, and there was little chance of development of an economically viable total synthesis, several pharmaceutical companies started screening for Taxol mimics from diverse natural sources, at first without any success in spite of the enormous numbers of compounds and extracts tested.[7] Colleagues at Merck, Sharp, and Dohme screened extracts from myxobacteria, having obtained access to the collection of *Sorangium* strains of Dr. John E. Peterson, a retired botanist and professor at Emporia State University in Kansas, who had worked on those organisms 30 years ago. They soon found the desired activity, and with the small quantities of substance they could recover from plate cultures they elucidated the structure, only to find out that they had rediscovered epothilone. They retained our name, just adding the "e," and cited our patent. The publication of their results, including the mechanism of action,[8] which we had failed to determine, started an avalanche of patents and publications on total synthesis, biosynthesis, genetics, molecular biology, biochemistry, and preclinical and clinical work, which still is increasing in volume at a rapid pace,[9] including numerous reviews.[10]

It soon turned out that epothilone has several properties that could make it superior to Taxol. It acts at very low doses (in the nano- to picomolar range), is active against multidrug-resistant (MDR), including Taxol-resistant, cancer cells, and is more water soluble, so that certain derivatives can even be administered orally. Further, it can be produced by fermentation without difficulty, and as a smaller and simpler molecule, is also accessible by total synthesis, enabling the production of many variants (so far about 1000).

The epothilone story following publication of the 1995 Merck article[8] is a good example of the often-overlooked fact that basic research and its reduction to practice is far from a straightforward process. When the Merck group discovered our patents, they terminated the project without contacting us regarding possible collaboration. Later, we ourselves tried to interest four major German, one Swiss, and three U.S. pharmaceutical companies in the possibility of collaboration in epothilone development, supplying test material to each company. After over 18 months of consideration, we finally came to an agreement with Bristol-Myers Squibb. The Swiss (Novartis) and one of the German companies (Schering) soon started independent epothilone projects, Schering using total synthesis, and Novartis with material produced by fermentation, using our patent strain, So ce90. By 1997 we had already developed fermentation and separation processes with mutant strains, allowing us to produce 10 g amounts of pure epothilone A and B.[10c] The yields could later be increased using traditional methods to several hundred milligrams per liter, so that, contrary to

1985	Dr. Reichenbach at GBF isolates *Sorangium cellulosum* strain So ce90 from a soil sample
1987	The strain is screened positive for antifungal activity, epothilone A and B were isolated, and their structures elucidated by Dr. Gerth and Dr. Bedorf
1991	Immunosuppressive activity studied by Ciba Geigy First publication of structure in Sci. Ann. Rep. of GBF
1992	Application tests and field trials for plant protection by Ciba-Geigy
1994	Work on epothilones terminated at GBF, international patent application abandoned Good activity in NCI 60 cell line antitumor screening
1995	Rediscovery of epothilone A and B as taxol mimics at MSD Disclosure of the absolute configuration as personal communications by Dr. Höfle
1996	First total syntheses by the Danishefsky, Nicolaou, and Schinzer groups
1997	Large scale production at GBF by fermentation Joint semisynthesis program started at BMS and GBF
1999	Clinical trials with natural, synthetic, and semisynthetic epothilones commence
2000	Biosynthesis genes cloned by Novartis and Kosan
2003	Phase II/III clinical trials with epothilone B-lactam (Ixabepilone)

FIGURE 21.2 Timeline for epothilone discovery and development.

statements in the literature, it was possible to produce all the material needed for development and future applications using *Sorangium cellulosum*, which can be cultivated without problem on cheap media based on soy meal in industrial bioreactors on a 60–100 m^3 scale. The time course of epothilone discovery and development is shown in Figure 21.2.

II. NATURAL EPOTHILONES

The epothilones were discovered in cultures of the myxobacterium *Sorangium cellulosum*. Myxobacteria are Gram-negative bacteria that move by gliding along surfaces and are notable for their highly developed intercellular communication systems and their ability to reproduce, under unfavorable living conditions, in a cooperative action involving hundreds of thousands of cell fruiting bodies of a remarkably sophisticated shape and structure.[11,12] The producing organism (Figure 21.3), strain So ce90, is a cellulose degrader and was isolated at the GBF in 1985 from a soil sample collected on the banks of the Zambesi river in southern Africa. In addition to epothilones A and B, the strain synthesized in large scale a family of novel spiroketal polyene polyketides, named spirangiens,[1,13] which also showed antifungal traits and cytotoxicity. The epothilones aroused immediate interest because of their strong immunosuppressive effect and good activity against oomycetes — important parasites of agricultural plants.[5] Both effects lacked practical applications, however, because of the high toxicity of the compounds. The need for gram amounts of epothilones for relevant tests had already led at that time to the establishment of a production and isolation process at the GBF; however, because of the presence of an excess of spirangiens, the isolation of pure compounds was cumbersome and only achieved by multistep chromatography.

The discovery in 1995 that *S. cellulosum* strain SMP44 also synthesizes epothilones made a second producer strain available.[8] This strain is used for practically all studies in the Unites States, with the exception of the BMS/GBF project. Epothilone production with SMP44 was performed in large (150 mm) Petri dishes on agar for 10 d at 28°C. From 80 plates, 2.7 mg of pure epothilone A and 0.9 mg of epothilone B could be recovered, which was a sufficient amount to elucidate the structures by nuclear magnetic resonance and mass spectroscopy.[8] Similarly, the initial yields of epothilones from the GBF strain were rather low. As a first step in yield improvement, strain So ce90 was ~~mutated~~ to eliminate spirangien synthesis, which competed for precursors (the first cloned spirangien negative clone was obtained in October 1996).[14] This was possible only after the strain

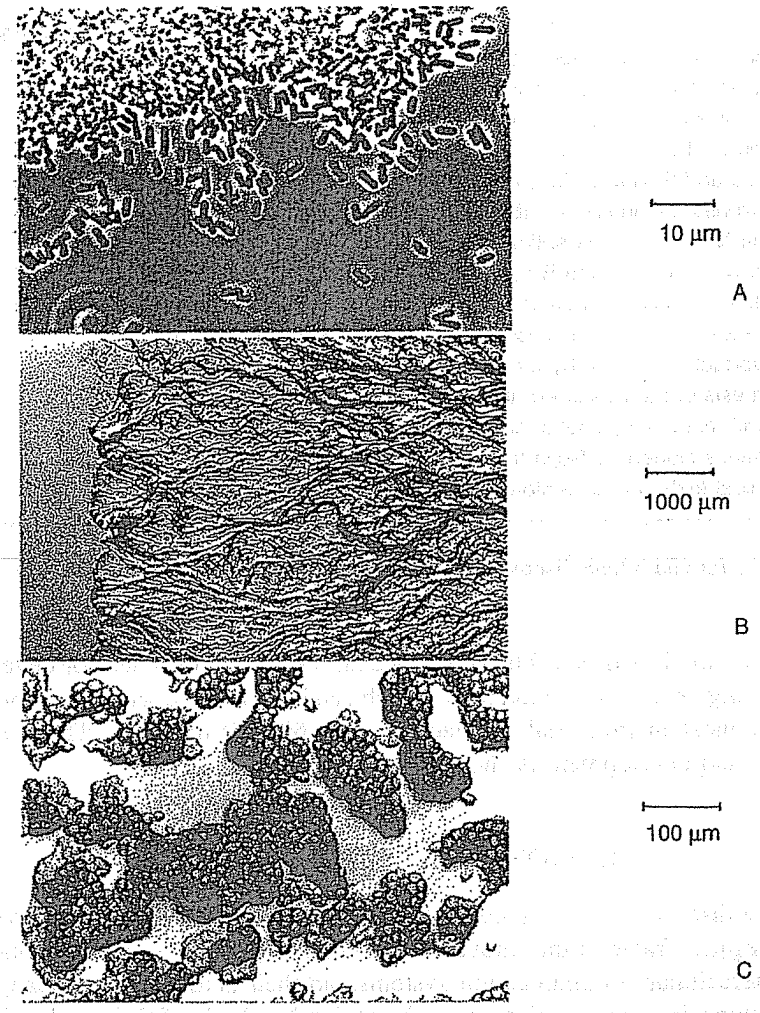
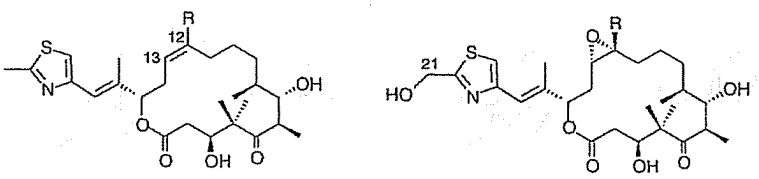


FIGURE 21.3 The myxobacterium, *Sorangium cellulosum*, producer of epothilone. (A) Vegetative cells; (B) vegetative, spreading swarm colony, (C) fruiting bodies, consisting of tiny sporangioles.

had been adapted to grow in liquid media in single cell suspension and to develop colonies from individual cells after plating, which are both time-consuming procedures in myxobacteria. A first optimization of the fermentation medium was then undertaken.

Cellulose-degrading *S. cellulosum* is much more versatile metabolically than most myxobacteria, notably *Myxococcus xanthus*, and grows on complex as well as on very simple media. Our first production medium consisted of defatted soybean meal, potato starch, glucose, yeast extract, and mineral salts.[2] As epothilones are excreted into the broth, a hydrophobic adsorber resin, Amberlite XAD-16, was added during fermentation. The resin increased yields by a factor of five by avoiding feedback inhibition by the end product, and by prevention of its degradation.[9,15] The medium was later improved, among others, by adding skim milk powder.[16] Production was during log and early stationary phase, with yields of 22 mg/L epothilone A and 11 mg/L epothilone B.[2] The generation time was around 16 h, and cell densities of up to 2×10^9 were achieved. After 7 d of fermentation, the resin was harvested by sieving, and the epothilones were extracted with methanol and processed further. Later, the yields could be increased substantially by further optimizing the medium, and especially by a mutation program during which more than 24,000 mutant clones were generated by treatment either with ultraviolet light or nitroso guanidine (NTG)



Epothilone C 2a R = H
Epothilone D 2b R = CH_3

Epothilone E 3a R = H
Epothilone F 3b R = CH_3

FIGURE 21.4 Structures of the minor epothilones.

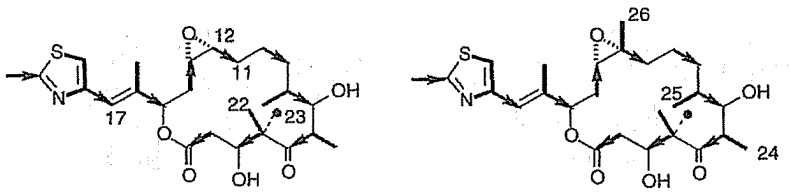
and were characterized by high performance liquid chromatography analysis.[17] Thereby, not only high producers but also clones with defective biosynthetic pathways were obtained,[15–17] among them strains that produced epothilone A only, but none that produced pure epothilone B. The same was observed with about 40 further epothilone producers discovered among the 1700 *S. cellulosum* strains of the GBF collection. The positive strains came from soil samples collected on four continents and represented between 1% and 2.5% of all strains, depending on the area of origin.[9] The various strains differed not only in the epothilone pattern but also in the physiology of production. Thus, for example, epothilone synthesis is stimulated by glucose in So ce90 but inhibited in strain So ce1198.[9] So far, epothilones have not been found in any other myxobacterial species, nor in any other organism.

From high-producing *S. cellulosum* strains, small amounts of epothilones C (**2a**), D (**2b**), E (**3a**), and F (**3b**) (Figure 21.4) were isolated, in addition to epothilones A and B.[4,18] Whereas in C and D the epoxide is replaced by a cis double bond, an additional hydroxyl group is present at the thiazole methyl group in the variants E and F. Later, both desoxyepothilones **2** and hydroxyepothilones **3** became available by fermentation of a P450 defective mutant and by biotransformation with *S. cellulosum*, respectively. This greatly facilitates their isolation, but separation of the homologous pairs still requires careful reversed-phase chromatography.

From a large-scale fermentation (700 L) of So ce90 mutant B2, 35 variants could be isolated in very small amounts in addition to epothilones A–F.[4] Of those, only epothilones C_7 and C_9 may be regarded as regular biosynthetic products carrying extra hydroxyl groups at C14β and C27. The majority of the other variants result from aberrant biosynthesis. They lack methyl groups, carry extra methyl groups, have an oxazole instead of a thiazole ring and extra double bonds, and have reduced (by two C-atoms) or expanded ring sizes (epothilones K and I). Several small open-chain molecules are obviously derived from biosynthetic intermediates that had escaped from the enzyme complex.[4]

The epothilones A–F are colorless solids, readily soluble in polar organic solvents and stable at ambient temperature in the pH range of 5–9. Epothilones A, B, and D are crystalline, with melting points in the range of 76°–128°C, depending on the solvents of crystallization (the correct melting point of epothilone A is 76°–78°C [ethyl acetate/toluene], and 85°–87°C [methanol/water].[3,19–21] Several crystal structures are available for crystals from lipophilic and polar solvents exhibiting different conformations (Figure 21.7; Section V).[3,20] Aqueous solubility depends crucially on the presence of epoxide and C12 methyl groups and ranges from 700 mg/L for epothilone A and 200 mg/L for B to 16 mg/L for epothilone D.[3,21] Thus, epothilone D is about as poorly water soluble as paclitaxel (6–30 mg/L),[22] posing similar problems with its administration at therapeutically useful doses. Nevertheless, because of its promising therapeutic window, epothilone D was identified very early as a candidate for clinical studies by the Danishefsky group.[23] Total synthesis was upscaled and production by fermentation optimized. Under favorable conditions, our So ce90 P450 defective mutant produces 70 mg/L of epothilone D.

At Kosan Biosciences, the genes for epothilone synthesis were cloned from strain SMP44, first into *Streptomyces coelicolor*[24] and then into another myxobacterium, *M. xanthus*.[25] Both strains, however, produced less than 0.2 mg/L of epothilones. By eliminating the P450 epoxidase gene,



Epothilone A Epothilone B

FIGURE 21.5 Incorporation of acetate, propionate, methionine, and cysteine in epothilones A and B.

clones that produced only epothilones C and D (0.45 μg/mL) were obtained.[26] Later, the yields could be increased to 23 mg/L and to 90 mg/L[26,27] by optimizing the fermentation process. This process requires the addition of methyl oleinate, which complicates work-up; thus, only 63 mg/L of epothilone D could be recovered.

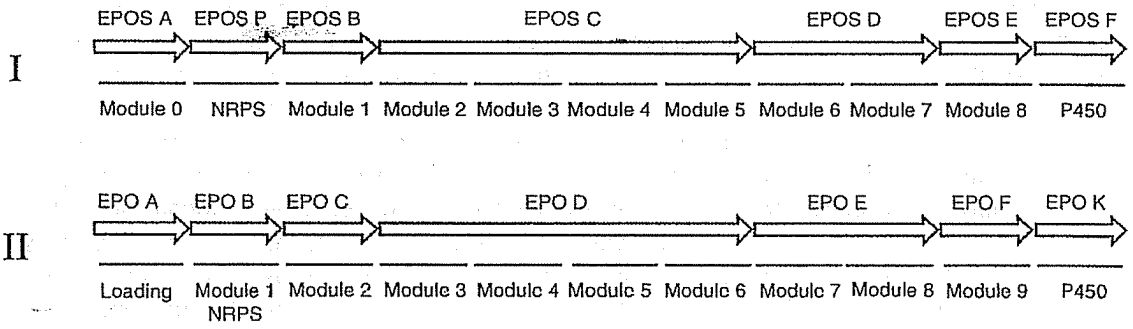
Biogenesis of epothilones has been studied in *S. cellulosum* on three levels: actual biosynthesis using feeding experiments and blocked mutants; analysis of the genes coding for the relevant enzymes; the genes were cloned and sequenced from both producer strains, So ce90 and SMP44; and *in vitro* synthesis using part of the enzymatic apparatus cloned and expressed in *Escherichia coli*.

By feeding ^{13}C and radioactively labeled precursors to strain So ce90, it was quickly established that the carbon chain is synthesized from acetate, propionate, and cysteine (Figure 21.5; the correct stereochemical assignment of the 22-Me and 23-Me groups is given in references 184 and 185).[16] Methyl C21 comes from acetate, methyl C22 from S-adenosyl methionine, and all other methyls arise from propionate. This is also the case with methyl C26 at the epoxide site. The incorporation of propionate at this site in place of acetate is a result of a low specificity of the enzyme in this region.

So far it has not been possible to isolate strains or mutants that synthesize exclusively the more active epothilone B, although strains restricted to epothilone A are available.[16] Synthesis starts by joining cysteine and acetate to yield methyl thiazole. This requires participation of a nonribosomal peptide synthetase (NRPS). The rest of the molecule is essentially provided by polyketide synthase (PKS) modules.[16] As was corroborated by the genetic approach, the whole biosynthetic machinery consists of a huge hybrid multienzyme complex composed of one NRPS and eight PKS modules. The oxygen of the epoxy group is introduced by a post-PKS monooxygenase using molecular oxygen. The PKS module responsible for this section of the molecule has, however, no dehydratase sequence that could produce the double bond required as the substrate of the monooxygenase. As we could isolate two epothilone variants, one with a hydroxyl on C13 and the other with a C12/C13 double bond, which is perhaps produced by a dehydratase (DH) in another module, the substrate of the monooxygenase may arise in this way.[17]

The dehydration must happen at an early stage because the hydroxy precursors of epothilones C and D could not be detected.[17] Increasing amounts of epothilones A or B in the medium inhibit *de novo* synthesis of those molecules. At the same time, epothilones C and D, present only in traces in the control, rise to very high levels.[17] Obviously, the monooxygenase responsible for the introduction of the epoxy group is feedback inhibited by epothilones A and B, and epothilones C and D are the end products of the PKS. When epothilones C or D are fed to the nonproducing So ce90 C2 mutant, the compounds are bound immediately to the cells and transformed into epothilones A/B. The monooxygenase is found only in epothilone-producing strains and was never seen in other *Sorangium* strains.[17] It appears that epothilone C has a higher affinity for the monooxygenase than epothilone D, as it is preferentially epoxidized, and even slight and remote changes in the molecule may prevent epoxidation. The block mutant D48 is defective in its monooxydase and is thus a producer of epothilones C and D.[17]

In cultures of So ce90 without XAD resin, yields of epothilones A/B go down dramatically, and a small proportion of the material becomes hydroxylated on C21 to give epothilones E/F.[15] Degradation starts with the opening of the lactone ring by an esterase, which can be demonstrated



SCHEME 21.1 Analysis and assignment of epothilone biosynthesis genes by the Novartis (I) and Kosan groups (II).

in the culture supernatant. The hydroxylation is performed by another epothilone A/B–induced monooxygenase, which appears to be a P450 enzyme, for it is inhibited when iconazole is added to the culture.[15] A screening of 95 wild strains of *S. cellulosum* showed that this monooxygenase is widely distributed among nonproducing *Sorangium* strains.

Comparable biosynthetic studies are not available for strain SMP44 and the heterologous host, *M. xanthus*. For those strains, it was shown that the composition of the medium may have an influence not only on epothilone yields but also on the pattern of variants produced.[28,29] In *M. xanthus*, addition of serine resulted in a substantial increase of epothilones that have an oxazole instead of the thiazole ring. Oxazole epothilones are normally found only in trace amounts, also in So ce90 fermentations.[4] Their synthesis is obviously a result of a lack of absolute specificity of the NRPS module taking occasionally a serine instead of a cysteine precursor. The epothilone genes have been cloned and sequenced from both producer strains, So ce90[30] and SMP44.[31] Both sequences are patented.[32,33] As mentioned, the SMP44 genes have been modified and used to produce epothilones heterologously.

Good summaries of the organization of the epothilone gene cluster are available[30,31,34] (Scheme 21.1), and because of space limitations, details can not be discussed here. Briefly, in SMP44, all seven genes required for epothilone synthesis, *epoA* to *epoF* and *epoK*, are clustered together in a 56–kilobasepair stretch of DNA.[31] *EpoA* is the loading domain, *EpoB* the NRPS module, and *EpoC* to *EpoF* are PKS modules. Overall, there are nine modules plus the loading domain and *EpoK*, a P450 monooxygenase responsible for the introduction of the epoxy group. All genes are transcribed in one direction and probably in one operon. A methyl transferase domain in module 8 (EpoE) may be responsible for the introduction of one of the methyl groups on C4. When EpoK was expressed in *E. coli*, the isolated protein could be used to epoxidize epothilone D into epothilone B.[31] There is an anomaly in the sequence of PKS module 4: the DH required for the introduction of the C-12/13 double bond of the PKS end product is lacking. The hypothesis that the DH of the neighboring module 5 may be responsible for creating that double bond has recently been proven by destroying that domain.[35] Although the exact mechanism of the enzymatic reaction is not yet elucidated, a forward–backward shuffling of the growing chain beween modules 4 and 5 is suggested. Another anomaly is found in module 9 (*Epo F*), where the DH and ER are inactive[31] ([3-^{13}C, 3-^{18}O] labeled epothilones were isolated after feeding of [1-^{13}C, 1-^{18}O] propionate[21]).

The group sequencing the So ce90 epothilone genes used a slightly different nomenclature for the same general organization of the gene cluster[30] (Scheme 21.1). In this case also, transcription is unidirectional. It is suggested that all those genes form one operon of more than 56 kilobasepair. The finished polyketide, epothilone C/D, appears to be released from the PKS by a thioesterase; a sequence showing conserved motifs of that enzyme has been identified next to module 8 in *epoE*.[30] A methyltransferase domain in *epoD* is required to produce one of the gem methyl groups at C4. The sequence of *epoF* indicates a cytochrome P450 oxygenase. A short distance in front and behind

the epothilone genes, two ORFs, *orf3* and *orf14*, seem to code for transport proteins that may be involved in export of epothilone from the producing cell.[30] In summary, the genetic apparatus for epothilone synthesis is remarkably similar, in fact, almost identical, in two producing strains of *S. cellulosum,* although these are of completely different origin. Among 56,000 basepairs, only 407 (= 0.7%) differ in the two gene clusters, which is an astonishing and very unusual correspondence not yet seen with any other *Sorangium* compounds (Rolf Müller, personal communication).

The epothilone gene cluster from SMP44 was cloned and expressed in heterologous hosts that actually produced epothilones.[24,25] The *M. xanthus* system was also used to make structural variants by manipulating the gene cluster. Thus, by inactivating the monooxygenase gene, strains were obtained that synthesized preferentially epothilone D.[25–27] Using site-specific mutation, epoD strains were further modified by inactivating the enoyl reductase domain in module 5 to make 10,11-didehydro-epoD (=Epo490),[36] and by inactivating the ketoreductase domain in module 6 to synthesize 9-keto-epothilone D.[37a] In addition, 4-desmethyl epothilones, molecules with an additional ether ring, and two fragmentary epothilones were obtained.[37a] Recently, it was demonstrated that an *E. coli* strain engineered to express the last half of the epothilone biosynthetic pathway is able to process an advanced precursor to epothilone C.[37b] In the future, this approach possibly may allow for the exclusive production of epothilone D and its analogs from simple synthetic precursors, thus avoiding tedious separation of C/D homologs. By biotransformation using the oxygen-introducing bacterium, *Amycolata autotrophica* (formerly *Nocardia autotrophica*), hydroxylated variants were produced from epothilone D and 10,11-didehydro-epothilone D.[38] Several of the analogs created by those different methods are also known from So ce90 fermentations.[4] The genes of the first three modules of the cluster, *EpoA*, *EpoB*, and *EpoC*, were expressed in *E. coli*, the resulting proteins purified, and their enzymatic reactions studied *in vitro*.[34,39–43] Products were usually obtained in minute quantities but could be identified by comparison with synthetic material. The system is of particular interest because it allows us to study PKS/NRPS/PKS interfaces in this hybrid multienzyme complex. It turned out that the individual enzymes of the epothilone cluster show considerable flexibility with respect to their substrate specificity, which could be useful for combinatorial biosynthesis. However, before application, the problem of producing reasonable yields has to be solved.

In a recent study, it was demonstrated that the base sequence of the KS domain of *epoB* in So ce90 (corresponds to *epoC* in SMP44) differs in several respects from the KS domains of all other epothilone modules.[44] Although those, in a phylogenetic analysis, cluster closely together, as is the rule in PKS genes, the *epoB* KS goes with PKS modules of other organisms. Also, the *epoB* KS deviates significantly in its GC composition. This was taken as a hint that the *epoB* KS sequence may have been introduced into a rudimentary epothilone gene sequence by horizontal gene transfer. Also recently, the exact position of epothilone bound to a protein could be analyzed for the first time.[45] The crystal structure of the P450epoK monooxygenase was analyzed in the free form and again with the substrate and product bound. The enzyme has a very large binding cavity, but otherwise it resembles other P450 enzymes, particularly P450eryF from the erythromycin cluster (30% identity, 48% similarity).

III. MECHANISM OF ACTION

The biological and biochemical effects of the epothilones have been studied in many laboratories, applying diverse experimental methods, in the United States and in Europe, and results are not always strictly comparable. Clearly, individual cell lines and the various epothilone analogs behave quite differently. Nevertheless, the overall picture is fairly consistent, and as a rule, safe generalizations can be made. In the ensuing discussion, frequent reference is made to synthetic and structure-activity studies discussed in detail in Sections IV and V, respectively.

Epothilone does not act on bacteria, and among the filamentous fungi and yeasts tested, only *Mucor hiemalis* (Zygomycetes) responded (minimal inhibitory concentration 20 μg/mL).[2]

In greenhouse experiments, various oomycetes were also inhibited. The cytotoxicity of the epothilones in animal cell cultures was noted immediately after their discovery,[1,2,5] but the effects they produce on the tubulin cytoskeleton were only realized much later.[8] In the beginning, only the two main fermentation products, epothilones A and B, were available for study. Although epothilone B differs only in an additional methyl group on C13, its efficacy is consistently higher by a factor of 5–10.[4,8,10e,m,46–51] This difference also remains with analogs of the two (e.g., epothilones C and D[4,17,46,49,52] or the oxazole derivatives[4,53,54]) and is even seen in binding and polymerization experiments with tubulin.[47] The epothilones act on a wide variety of cell types, including many human tumor cell lines. The concentration range for cytotoxicity is IC_{50} = 0.3–4 ng/mL[10n] or 1.3–39 n*M*[8,10m,46–48] for epothilone A, and 0.1–1.5 ng/mL[4] or 0.06–40 n*M* for epothilone B.[8,10m,46–48] The figures for Taxol are 0.2–95 n*M*.[8,46,47] (The molecular weights are Taxol, 854; epothilone A, 494; epothilone B, 508; i.e., Taxol is 1.73 and 1.68 times larger than epothilone A and B, respectively). Efficacy of Taxol equals that of epothilone A and is clearly less than that of epothilone B.[8,46,47] For instance, with 0.05–0.06 vs. 0.22–0.6 n*M*, epothilone B is 6–10-fold more active than Taxol against human prostrate cancer cells.[52] It came as a big surprise and is of considerable practical relevance that the epothilones still act on Taxol-resistant and MDR cells in general.[8,47] Although the resistant cell lines tolerated 100- to several thousand–fold higher concentrations of the selective agent, such as Taxol, vinblastine, or adriamycin, resistance to epothilones rose only by a factor of 1.5–10, and rarely higher. For epothilones C and D, it was even found to decrease in some instances.[46] It was reported that epothilone B–lactam and epothilone C–lactam lose activity on drug-resistant cell lines,[48,55,56] but this was not corroborated by another study, using several drug-resistant human cancer cell lines.[57] *In vivo* studies also did not support that statement. Epothilone B–lactam indeed shows about the same activity as epothilone A. Resistance to epothilone itself will be discussed below.

The epothilones promote tubulin polymerization and stabilize microtubules (MTs), as does Taxol. The morphological changes produced in treated cells resemble those effected by Taxol. In interphase cells, massive bundles of MTs arise in the cytoplasm, apparently randomly and independent from centrosomes, originating from multiple nucleation centers.[8,47,58,59] This transformation can already be seen 2 h after application, and after 24 h, virtually all MTs are in bundles (epothilone B).[47] When cells are kept at 4°C, their MTs decay completely within 1 h, but in the presence of 25 μ*M* epothilone A or B or Taxol, they remain stable.[8] Already, 2 h after the addition of epothilones, mitotic defects can be observed, reaching a maximum after 6 h.[47]

At concentrations of 10 μ*M*, normal spindles are no longer seen.[47] The organization of MTs in mitotic cells differs from that in interphase cells, so that a considerable turnover of tubulin still must occur in spite of the MT-stabilizing effect of the drug.[8,47] An explanation could be that before mitosis, MT-destabilizing proteins, such as katanin or stathmin/op18, are activated, or that MTs are stabilized less rigorously, perhaps because MT-associated proteins (MAPs) have dissociated after having become phosphorylated.[60] Multipolar spindles arise in a concentration range between 5 n*M* and 1 μ*M*.[8] The spindles produced in the presence of epothilone A, epothilone B, and Taxol seem to differ somewhat in their morphology.[47] The result of those mitotic aberrations is an arrest of the cell cycle at the G2–M stage (e.g., with 100 n*M* epothilone A or B after 72 h).[8,47,52,57] The DNA content is then 4n.[52] The mitotic blockade is complete after 8 h (at the ID_{50}, 7.5 n*M* of epothilone B–lactam,[57] or in another system, after 20 h at 3.5 n*M* epothilone B[60]). Mitotic arrest and cytotoxicity are seen at much lower doses than aberrations in interphase MTs (e.g., at 3–30 n*M* vs. 6–40 n*M* vs. more than 1 μ*M*, respectively).[8] A certain percentage of cells seem to pass through aberrant mitosis and become arrested in the following G1 phase as multimininucleate cells.[8] With epothilone B at its IC_{50}, 38% of cells become multimininucleate, and with epothilone A and Taxol, 10%–15% do so. Delayed mitosis takes place in those cells 24–30 h after the addition of the drug, and although they die, too, they do so after a longer time. The phenomenon is preceded by an aberrant chromosome alignment in metaphase and is probably related to the apoptotic fragmentation of nuclei discussed below. In MCT7 cell cultures, 2.5% of control cells are in

mitosis.[60] When epothilone B is added, many cells enter mitosis but do not progress beyond metaphase. The ratio of anaphase to metaphase cells in the control was 0.18; with 1 n*M* epothilone B, 0.03; and at 3 n*M*, 0. Many of the cells that were not blocked in mitosis became multinucleate, probably because of an abortive mitosis; the behavior of cells exposed to epothilone B at a dose too low to lead to mitotic arrest is of considerable interest. In this case, multipolar spindles are induced, resulting in aneuploid cells which also are not viable.[61]

The epothilones lead to apoptosis, as indicated by DNA ladders. The DNA is nicked specifically in G2–M blocked cells.[8] Further, fragmentation of the cell nuclei is seen. This can be monitored automatically by an image analyzer.[62] With L926 mouse fibroblasts, fragmentation of nuclei can be seen already after 1 d and reaches its maximum after 3 d (100 ng/mL epothilone B). Fragmentation increases linearly with the logarithm of concentration between 1 and 1000 ng/mL epothilone A.[62] Treated cells appear to remain vital for some time, at least the reduction of tetrazolium salts continues in mouse macrophages unrestricted for 48 h (epothilone B, 0.02–20 ng/mL).[59]

A comparison of epothilones B and D showed that total uptake of epothilone B is higher by a factor of 4.[63] In addition, the intracellular distribution of the two compounds differs dramatically. Although nearly all of epothilone D is found in the protein fraction of the cytosol, almost 50% of the epothilone B resides in the nucleus.[63] This different behavior was corroborated by a more detailed study using tritiated 6-propyl-epothilones B and D (pEpts B and D), which show almost the same inhibitory effects as the natural 6-methyl analogs.[64] The study compared two cell lines, A431 (human epidermoid) and NCI/Adr (human mammary carcinoma, Taxol resistant up to more than 1μM). Epothilone D and pEpt D acted much more slowly than epothilone B and pEpt B, which produced almost maximal and lasting growth inhibition after only 4 h exposure in A431 cells. Drug uptake and intracellular pools turned out to be dose dependent. Efflux of pEpt D was faster (almost complete after 60 min) than that of pEpt B, 60%–70% of which was still inside the cells after 4 h when the loading dose was high (70 n*M*). When it was low (3.5 n*M*), NCI/Adr, in contrast to A431 cells, lost pEpt B quickly. This demonstrates that the two cell lines have different transport mechanisms for the two analogs. Apparently, the export pump of NCI/Adr cells becomes blocked when pEpt B is present in high concentrations.

In both cell lines there seems to exist a MDR1-independent, yet verapamil-sensitive, export. Verapamil, a Ca antagonist and inhibitor of the MDR1 P-glycoprotein (Pgp) shuttle, stimulates uptake of both drugs at low drug concentrations, but at high concentrations stimulates uptake only of pEpt D. Intracellular pools may reach up to 100 pmol/10^6 cells. Drug distribution was the same in both cell lines: pEpt D (and Taxol) was mainly in the soluble part of the cytosol (80%), and only little was in the nucleus (5%–15%), whereas half of the pEpt B was in the nucleus (40%–50% vs. 40%–60% in the cytosol); there it was located on nuclear proteins, presumably tubulin, and nothing was on the DNA. This distribution may contribute to the differences in drug transport and efficacy of the two analogs. The influence of epothilone B on MT dynamics was directly measured on time-lapse photographs of MTC7 cells that were stably transfected with green-fluorescent-protein α-tubulin.[60] Cells were analyzed 6 h after the addition of the drug, when the intracellular equilibrium was reached.

The growth rate of MTs in control cells was 13.3 μm/min, the shortening rate 21 μm/min, and dynamicity (length of growth + length of shortening during total observation period per minute) 10.4 μm/min. With 2 n*M* epothilone B, those figures were reduced to 8.3, 19.3, and 6.3 μm/min, and with 3.6 n*M*, to 7.5, 14.9, and 3.9 μm/min. Thus, while under all conditions MTs shortened twice as fast as they grew, epothilone B clearly reduced MT dynamics. Also, at 3.5 n*M* most MTs were completely stabile, although their ends showed minute (below 5 μm/min) changes in length (they "chatted"). The few MTs that still were dynamic grew and shortened more slowly and for shorter lengths of time; also, pause times, at which no changes occurred, became longer. The concentration and timescales of the MT effects correlate closely with those of mitotic arrest, and it was concluded that reduction of MT dynamics is the direct cause of mitotic arrest.

Spindle MTs may be particularly sensitive to interference with their dynamics because they have to grow for a longer time and to greater length than interphase MTs. Essentially the same reactions as just described for epothilone B were seen with Taxol, only at somewhat higher concentrations. However similar the reactions of cells to Taxol and epothilone are, they still are not identical. An example is the behavior of mouse macrophages (*in vitro*).[59] At very high doses (0.1–10 μg/mL) Taxol stimulates the macrophages to produce endotoxin-like effects; for example, to synthesize nitric oxide (*Salmonella typhimurium* lipopolysaccharide does so at 1–10 ng/mL). Epothilone B (up to 10 μg/ml) neither induces macrophages to release nitric oxide nor prevents it when they are treated with lipopolysaccharide, yet epothilone B acts normally on MTs inside the cell (at 12.5 ng/mL). Also, the degrees of resistance of normal cells to epothilone and Taxol do not always match.[65]

Studies on the effects of epothilones on tubulin *in vitro* showed that they make tubulin polymerize under conditions not applicable in the absence of the drug; for instance, in the absence of GTP[8,47] or MAPs or both, and in the presence of Ca^{++} (3 m*M*)[8] and at low temperatures (0°C).[47] They stabilize existing MTs at cold temperatures (4°C for 30 min,[8] 0° for at least 1 h[47]), with epothilone B doing so even after the addition of 5 mM $CaCl_2$ (not, however, epothilone A or Taxol).[47] Although initial studies suggested that epothilone A is more effective than epothilone B,[8] this was later corrected when purified tubulin was used.[47] The observed effects resemble those produced by Taxol, and indeed it was shown that epothilones displace [^{3}H]-Taxol from polymerized tubulin[8,47] (apparent K_i values, depending on the method of data analysis: epothilone A, 1.4/0.6 μ*M*; epothilone B, 0.7/0.4 μ*M*; docetaxel, 1.2 μ*M*).[47] This indicates both that the epothilones dock to the binding site of Taxol (i.e., β-tubulin), although initially allosteric effects or overlapping binding sites could not be excluded, and that epothilone B binds more tightly than epothilone A and Taxol. More thorough studies showed that epothilone A is close to Taxol in efficacy while there are still subtle differences.[47] Thus, for example, in experiments without GTP but with MAPs, epothilone A causes better polymerization than Taxol (both epothilones allow assembly at 10°C, maximum at 25°C, whereas Taxol produces no MTs below 25°C, maximum at 37°C), or with GTP but without MAPs at 25°C, Taxol is more active than epothilone A (epothilone B is already near its maximum) and reaches its maximum at 37°C, when it is twice as active as epothilone A. In the absence of both MAPs and GTP, significant polymerization takes place with all three drugs, only at higher concentrations (i.e., more than 10 μ*M* per 10 μ*M* tubulin, which was the ratio in the former experiments). Polymerization in the absence of GTP is somewhat enigmatic.

There are two GTPs bound to the tubulin dimer: one on α-tubulin at the α, β-interface, which is neither exchangeable nor hydrolyzable, and one near the carboxy end of β-tubulin that is exchangeable and becomes hydrolyzed to GDP soon after the addition of the heterodimer to the plus end of the growing MT (which is capped with GTP–tubulin). It appears that the GTP hydrolysis changes the shape of the dimer from straight to (in the free form) bean shaped.[66] This in turn may introduce some stress in the MT, produce the helical arrangement of the protofilaments, and may be a prerequisite for decay of the MT. Epothilone also reduces the number of protofilaments in MTs formed *in vitro* from 55% with 14 (control) to 67% with 13 (20 μ*M* epothilone B, 10 μ*M* tubulin).[66] The idea that the epothilones stimulate nucleation of tubulin polymerization is supported by two further experiments.[47] The critical concentration of tubulin, $[T]_{cr}$ (i.e., the lowest concentration at which polymerization begins under standard conditions), is regarded to be proportional to nucleation. The variable $[T]_{cr}$ was, without drug, 3 μ*M* and in the presence of any one of the three drugs, less than 1 μ*M*. In the presence of only MAPs or only GTP, $[T]_{cr}$, with Taxol or epothilone A, was 1.4–1.7/4.3–4.5 μ*M*, and with epothilone B, 0.8/2 μ*M*. Without both MAPs and GTP, $[T]_{cr}$, with Taxol, was 22 μ*M*, with epothilone A was 19 μ*M*, and with epothilone B was 7.4 μ*M*.

In addition, the average length of the MTs formed in the presence of the three drugs was measured in electronmicroscopic pictures. With MAPs and GTP but without drugs, the MTs were 4.3 μm long, with epothilones A or B, they were 2.8 and 1.1 μm, and with Taxol, they were 1.9 μm (i.e., shorter than with epothilone A). When GTP was lacking, the MTs became longer, and without MAPs even more so, yet with epothilone B, they were always half as long as with the

other drugs. The morphology of the MTs was the same with or without drug, viz, tubules with occasional ribbon-like open sections.

In summary, the experiments just described demonstrate that the epothilones bind to, or close to, the Taxol site on β-tubulin, that they (also) bind to polymerized tubulin, that they seem to stimulate nucleation, and that MAPs are not required for drug binding. There is an obvious discrepancy between the concentrations effective in producing cytotoxicity and mitotic arrest (lower nanomole range) and those interfering with MT turnover *in vitro* (micromole range). This may be explained by a several hundred–fold accumulation of epothilones inside the cells.[63,67] When HeLa cells, containing about 25 μ*M* tubulin, were exposed to 10 n*M* epothilone, the drug reached intracellular saturation levels after 2 h. The cells then contained 4.22 μ*M* epothilone A, or 2.55 μ*M* epothilone B, respectively. Exposure to 100 n*M* drug reversed the relative internal levels to 17 μ*M* epothilone A or 26 μ*M* epothilone B.[67] Thus, even at relatively low doses, both drugs reached intracellular concentrations sufficient to disturb MT dynamicity, which may lead to a lasting mitotic arrest after a short exposure of the cells to the drug.

In the last few years a much better understanding of the molecular interaction between Taxol/epothilone and β-tubulin has been gained. The yeast *Saccharomyces cerevisiae* is not inhibited by epothilone B (up to 150 μ*M*), which is likely a result of a lack of uptake of the drug,[68,69] as purified yeast tubulin is induced by epothilone B to polymerize *in vitro* (EC_{50} = 1.3 μ*M* in the presence of 5 μ*M* tubulin and 50 μ*M* GTP; with bovine brain tubulin, the EC_{50} is 1.2 μ*M*).[69] Interestingly, Taxol has no such effect. Epothilone B reduces the dynamicity of yeast tubulin by a factor of nine.[69]

In the presence of epothilone B, yeast tubulin also assembles without GTP. Yeast contains only one β-tubulin gene, and its β-tubulin differs in 124 amino acids from mammalian brain tubulin. With 75% similarity, it is close enough to coassemble into MTs. By analyzing available data on Taxol binding to bovine brain β-tubulin, five amino acids that differed in yeast tubulin were assumed to be responsible for preventing Taxol binding. Those amino acids were exchanged by site-specific mutation, viz, Ala19 to Lys19, Thr23 to Val23, Gly26 to Asp26, Asn227 to His227, and Tyr270 to Phe270.[68] The mutated yeast tubulin now polymerized with Taxol (EC_{50} 1.55 μ*M*, at 5 μ*M* tubulin under conditions that do not allow spontaneous assembly) even more efficiently than bovine brain tubulin, which is perhaps a consequence of the 5%–10% lower critical concentration of yeast tubulin. The EC_{50} of epothilone B remained the same (1.45 μ*M*) with yeast wild type and mutant tubulin. The molar binding ratio of Taxol:tubulin for wild-type yeast tubulin was 0.13, and for mutant tubulin it was 1.01, which was identical to that for bovine brain tubulin.[68] Radioactive Taxol bound to mutant MTs could be replaced almost completely by a 10-fold excess of epothilone B.[68] Clearly, the five inserted amino acids are not required for epothilone binding but are essential for interaction of Taxol with β-tubulin. This shows that the binding site of epothilone need not be exactly identical with that of Taxol. The unaltered EC_{50} of epothilone B for the mutant tubulin seems to indicate that epothilone indeed binds at a different location to Taxol.

Efforts have been made to identify the binding site of epothilone by mapping point mutations conferring resistance. The studies concentrated on the highly variable carboxy end of the otherwise conserved 450 amino acids of α- and β-tubulin, because this is the location of the nucleotide and MAP binding sites. Also, this part of the molecule is important for MT stability, because it is responsible for lateral contact of the protofilaments. In addition, most residues involved in Taxol binding have been located in this area. The mutants showed an increase in epothilone tolerance by a factor of 20 to 470.[70–73] Several of them had at the same time become hypersensitive to MT-destabilizing drugs (colchicine, vinblastine) or become outright epothilone dependent.[71,73] This was explained by a destabilizing effect of the mutation that is counteracted by the stabilizing activity of epothilone (or Taxol).[74]

The drug-resistant cell lines grow more slowly than the parent lines.[71] Expression of the MDR1 (Pgp) and the MPR (multidrug-resistance associated protein, another membrane protein) export systems are not increased[71,73] or even absent.[72] Also, there is no reduction of intracellular [^{3}H]-Taxol accumulation.[73] All this means that resistance is most likely caused by reduced epothilone

TABLE 21.1
Amino Acids in β-Tubulin Suspected to Be Involved in Epothilone Binding

Method	Responsible Amino Acids	Isotype	Reference
Resistant mutants	Thr274 to Ile, Arg282 to Gln		70
Resistant mutants	Pro173 to Ala, Gln292 to Lys, Tyr422 to Tyr/Lys (heterozyg.)	βI	71
Resistant mutants	Pro173 to Pro/Ala, Tyr422 to Tyr/Lys	βI	75
Resistant mutants	Ala231 to Thr, Gln292 to Glu	βI	73
Predicted from epothilone binding to P450epoK	Leu217, His229, Thr276, Arg284		45
Not required in yeast tubulin (in contrast to Taxol®)	Lys19, Val23, Asp26, His227, Phe270		68

binding. This has been shown to be the case by determining the percentage of tubulin polymerized with ~~the tubulin~~ decreased with increasing resistance from 28% (parental) to 6.9%.[73] In one cell line selected against 300 n*M* epothilone D, the mutated tubulin did not bind epothilone to any extent and also could not be stimulated to polymerize by the drug.[73] As a rule, epothilone resistance goes parallel to Taxol resistance,[73] but sometimes considerable discrepancies were observed — and even more so with respect to docetaxel.[70,71,73]

Usually, only one point mutation was discovered in resistant cell lines, but occasionally two could arise.[73] Most mutations were expressed on the protein level.[73] The identified amino acid exchanges were usually close to the M loop and on helices H7 and H9. Thus, the mutated residues were indeed near the Taxol binding site. Thr274 appears to be particularly critical for epothilone binding; it lies exactly at the site of interaction between β-tubulin and the C7–OH of Taxol.[72] However, the mapped mutations concerned a considerable variety of residues (see Table 21.1). This may be because epothilone resistance is a more complex phenomenon than it may seem at first look. For one thing, resistance may vary with different cell types, among others, because they vary in their β-tubulin isotype composition, including posttranslational modifications. Only recently it became possible to determine the exact isotype profile of an experimental cell line by liquid chromatography combined with electrospray ionization mass spectrometry.[75] This permits assurance that the isotypes present in the genome are really expressed under the conditions of the experiment, which is not necessarily the case.[76] The isotypes are encoded by different genes, and the various genes are preferentially expressed in specific cell types. They are more than 80% homologous and vary mainly at their carboxy ends. The isotypes differ in conformation, assembly, dynamics, and ligand binding.[63] Among the seven known isotypes of β-tubulin, I appears to be the preferred target of epothilone,[71,73,75] and II that of Taxol.[63] In HeLa cells, the prominent isotypes of β-tubulin were I and IVb. Two epothilone-resistant sublines contained the same isotypes with a separate heterozygous mutation, each in I. It could be shown that the mutant alleles were expressed, and apparently at a higher level than the wild type.[75]

Altered expression of isotypes[74] and changes in posttranslational modification of β-tubulin,[75] which could influence binding of MAPs, have to be considered as possibilities in producing resistance, although this has not yet been shown for epothilone. Nor can one be sure that tubulin mutations that lead to drug resistance are located in the β-isomer. In a Taxol-resistant, Taxol-dependent A549 cell line, the β1-tubulin was unaltered, and Taxol still bound to it, but there was a Ser379 to Arg exchange in the Kα1 isotype of β-tubulin.[74] Interestingly, there was an increased expression of βIII-tubulin, which also seemed to contribute to Taxol resistance, apparently by changing MT dynamicity.[74,75]

A major contribution to resistance may come from altered binding of MAPs to tubulin. MAPs may stabilize or destabilize MTs, and mutations in tubulin could influence the interaction between the two proteins, but of course, there could also be mutations in the MAPs themselves; in the

enzymes that activate or deactivate them, usually kinases and phosphatases; or in the systems that control the expression of MAPs. There is experimental evidence that those assumptions are not mere theory. In the Taxol-resistant (and Taxol-dependent) cell lines, A549-T12 and A549-T24, a rise in MT dynamicity is seen in the absence of Taxol.[74,75] In this case, the responsible mutation is in the α-tubulin. The point mutation is near the carboxy end and presumably at a site of MAP4 and stathmin interaction. In response to rising Taxol concentrations (2 n*M* is required for survival), the status of the two regulatory proteins is also changed. Whereas the active, hyperphosphorylated form of stathmin (which destabilizes MTs) was barely detectable, the intracellular level of the nonphosphorylated, inactive form increased twofold, and the phosphorylated, and therefore inactive, form of MAP4 (which is a MT stabilizer) had clearly risen.[74] In another study it was shown that tubulin-interactive drugs, like epothilone A, lead to phosphorylation of MAP4, probably by a mitotic kinase.[76] The phosphorylated MAP4 dissociates from the MTs, which then become disorganized. Drug-resistant cells do not phosphorylate MAP4, and their MTs remain intact with MAP4 associated.

At very high doses, however, the same events as in sensitive cells take place, which shows that the interaction between tubulin and MAP4 is undisturbed. Also, expression of MAP4 is not influenced by the drugs in sensitive and resistant cells. Thus, it appears that MAP4 takes part in the drug-induced killing process, and resistance is somehow connected with the failure to phosphorylate the molecule. Although the exact mechanism of this sequence of events is not yet understood, it is obvious that the binding of epothilone to tubulin activates downstream events that contribute to cytotoxicity.

In a more general approach, genes were identified by analyzing cDNA microarrays, which were overexpressed in epothilone A–resistant cell lines.[65,77,78] The STAT1 gene, interferon-inducible genes, and a MT-associated GTPase were found to be upregulated. The latter contributes perhaps to resistance by destabilizing MTs in the resistant cells. A recent study, also using cDNA microarrays, demonstrated that 41 genes had an altered expression after epothilone B (10 n*M*) treatment of cells, and that many of them were connected with the TNF (tumor necrosis factor) stress response pathway.[77]

Another important determinant in epothilone resistance could be the regulation of transport systems (i.e., of the membrane proteins involved in uptake and thus intracellular accumulation of the drug) and of the MDR1 and MPR machinery responsible for export. The latter are of particular interest in connection with epothilone, because they appear to be much less active on epothilone than on other cytotoxic drugs, including Taxol.

Studies on epothilone resistance repeatedly demonstrated that intracellular accumulation of tritium-labeled Taxol was unimpaired.[73] Thus, in those cases, resistance could not be attributed to impeded uptake of the drug. Similar experiments have been done with tritium-labeled epothilone analogs.[63,64] [^{3}H]-6-Propyl epothilones B and D were used to study uptake by nonresistant A431 (human epidermoid) and doxorubicin (Adriamycin)-resistant NCI/Adr tumor cells (which also are resistant to epothilones).[63] The resulting picture turned out to be rather complex, as pointed out already. In several epothilone B–resistant A549 and HeLa cell lines, the MDR1 gene was not expressed at all, and although the MRP system was expressed, its level of expression did not increase after the cells became epothilone resistant.[71] Also, in epothilone D–resistant leukemia cell lines, no rise in the levels of the MDR1 and MRP systems was seen, which could have explained resistance.[73] In fact, it already had been discovered that epothilones are not substrates for Pgp.[8,47] A human neuroblastoma cell line that was constitutive for MDR1 still responded to epothilone A, but not to Taxol.[58] The level of Pgp even rose during drug treatment (i.e., MDR1 was induced by epothilone A). In contrast to epothilone, calcein was exported from the cells ever more efficiently the higher the Pgp levels became, which proved that the transport system was fully functional. Thus, it seems that the cellular export systems do not play a major role in epothilone resistance, nor is there evidence that impaired uptake may be a decisive factor.

How does epothilone kill cells? All studies dealing with the killing mechanism of epothilone agree that the drug binds to MTs within cells, which leads to mitotic arrest by disorganization of the normal turnover of tubulin, to an accumulation of the cell population in the G2–M phase of the cell cycle, to a decay of the cell nuclei, and to apoptosis. However, there still remains to be

explained how exactly apoptosis is achieved, and whether apoptosis is the only way by which epothilones become cytotoxic. The answer is of more than academic interest, as it may have implications for the treatment of cancer. Thus far, no fully consistent picture can be presented. Among other reasons, this may be because investigators dealing with that problem used different cell types and various epothilone analogs.

It appears that the tumor suppressor protein, p53, does not play a role in the killing of cells by epothilone, as human neuroblastoma cell lines, which constitutively sequester p53 in the cytoplasm and thus prevent its effect as an intranuclear transcription factor, still were fully sensitive to epothilone A (and Taxol).[58] In contrast, it was shown that, although cells with mutated p53 normally become less sensitive to cytostatic compounds, their sensitivity to epothilone B (and Taxol) rises, perhaps because a higher proportion of cells with mutant p53 is in mitosis — the target of epothilone.[79]

Apoptosis is a rather complex phenomenon induced by a receptor-mediated extrinsic or a mitochondrial pathway, and it is modulated by a large number of proteins. Very briefly (for more details see, e.g., refs. 80 and 81), the extrinsic pathway starts with death receptors (e.g., DR4 and DR5) in the cell membrane. They are activated by binding a death ligand (e.g., Apo2-2L/TRAIL=TNF-related apoptosis-inducing ligand) and then, after several more steps, form a death-initiating signaling complex, which results in autocatalytic activation of initiator caspase 8, and the following effector caspase cascade finally triggers apoptosis.

The mitochondrial pathway begins with release of cytochrome c from mitochondria into the cytosol. This, which again involves several steps, leads to the formation of apoptosomes (consisting of oligomers of APAF-1 [apoptotic protease-activating factor 1]), which activate initiator caspase 9 and, in turn, executioner caspase 3 (and caspase 7). Those caspases cleave a number of cellular proteins and finally lead to apoptosis. The sequence is modulated by the Bcl-2 and IAP protein families. One study applying (water-soluble) epothilone B–amine (BMS 310705) on ovarian carcinoma cells taken from a patient who no longer responded to treatment with platinum and Taxol demonstrated that the drug killed the cells via the mitochondrial pathway of apoptosis.[80] The cells were exposed for 1 h to 10–500 n*M* of the drug and then incubated in drug-free medium. After 24 h, more than 25% of cells showed apoptosis, and after 96 h, 90% were dead. An increase in activity of initiator caspase 9 and effector caspase 3, but not of initiator caspase 8 (typical for the extrinsic pathway) was seen. Also, 12 h after treatment, cytochrome c was detected in the cytosol. Another study using epothilone B–lactam on cell cycle–synchronized human ovarian carcinoma cells found indicators of the mitochondrial pathway (leakage of cytochrome c into the cytosol, activation of caspase 3, and downregulation of the protective proteins survivin, cIAP1, and XIAP, which inhibit caspase 3, caspase 7, and caspase 9), but also a rise of the death receptors DR4 and DR5 in the cytoplasmic membrane, which sensitized the cells to the death ligand, Apo2-2L/TRAIL.[81] Thus, in this case the extrinsic pathway seemed also to be in operation. The study also showed that epothilone B–lactam is active on Taxol-resistant cells.

Still another study showed a role of the Bcl-2 family of apoptosis-modulating proteins in the killing process.[82] Again, epothilone B–lactam was used. The drug induced a conformational change in the proapoptotic Bax protein, which resulted in its transfer from the cytosol into mitochondria and a concomitant release of cytochrome c from mitochondria into the cytosol, with the consequences pointed out above. Apoptosis could be mitigated by overexpression of Bcl-2 and enhanced by the Bcl-2 antagonist, BAK-BH3 peptide. The conclusion was that epothilone B–lactam induces apoptosis by a Bcl-2-suppressible pathway, and that treatment of breast cancer can perhaps be improved by a combined therapy with epothilone B–lactam and a Bcl-2 antagonist. Involvement of the Bcl-2 family was also seen when human ovarian carcinoma cells were treated with epothilone A.[76] The epothilone A–tubulin interaction, which must be unimpaired, results in inactivation of the prosurvival proteins Bcl-2 and Bcl-x_L by phosphorylation, and in lowering of the Mcl-1 level by increased turnover. As Mcl-1 inactivates the proapoptotic Bax protein by forming a complex with it, reduced Mcl-1 and a concomitant overexpression of Bax clearly would support apoptosis. Typically, in resistant cells Mcl-1 remains high in the presence of epothilone A, Bax is lowered, and Bcl-x_L is not phosphorylated.

In contrast to the studies just discussed, the authors of an investigation of the effects of epothilone B on non–small cell lung cancer cells concluded that epothilone kills the cells by an as-yet-unknown, caspase-independent mechanism.[83] At low doses, cytotoxic effects (disruption of the MT cytoskeleton, arrest of cells in the G2–M phase, aberrant mitosis, multinucleated cells) are seen rather soon, before 24 h after the addition of the drug, yet symptoms of apoptosis [nuclear condensation, apoptotic bodies, cytochrome c release, cleavage of poly (ADP-ribose) polymerase, activation of caspase 3 and caspase 9] appear only much later, and therefore may be just side effects. Furthermore, the cells would still die when the mitochondrial or the extrinsic pathway of apoptosis were neutralized by overexpression of the antiapoptotic proteins, Bcl-2 or Bcl-x_L, or of the dominant negative FAS-associated death domain. Even the caspases could be blocked by natural (response modifier A) or artificial (Z-Val-Ala-Asp-fluoromethylketone) antagonists without interfering with cell death. If this hypothesis is correct, drugs may be found that induce that mechanism more efficiently and, thus, may be even more beneficial in cancer therapy.

Several studies report synergistic effects between epothilone and other drugs, which may become of interest in cancer therapy. Many solid tumors show interstitial hypertension, which impedes transfer of drugs from capillaries to tumor tissue. This high interstitial fluid pressure appears to be mediated by the activity of platelet-derived growth factor, the receptors of which are expressed in many solid tumors. The tyrosine kinase inhibitor, STI571 (Novartis Pharma), blocks the kinase activity of those receptors, which results in lowered interstitial fluid pressure and improves uptake of drugs by the tumor. When severe combined immunodeficiency (SCID) mice with human anaplastic thyroid carcinoma were treated with a combination of epothilone B and STI571, the tumors became more than 40% smaller than after treatment with epothilone B alone.[84] The level of epothilone B in the tumor increased threefold but remained unchanged in liver, kidney, and the intestinal tract. However, STI571 (100–150 mg/kg) had to be administered on three consecutive days at a minimum, and its effects faded away 2 d after the last application.

The synthetic flavonoid, flavopiridol, has aroused considerable interest in recent years on account of its antitumor activity. It arrests cells in the G1–S (or G2–M) phase of the cell cycle and induces apoptosis. The mechanism appears to be inhibition of mRNA synthesis, perhaps by blocking transcriptional elongation or by binding to duplex DNA. In consequence of this, cyclins and antiapoptotic members of the IAP and Bcl-2 families of proteins are downregulated.[85] Further, flavopiridol blocks cyclin-dependent kinases and brings about conformational changes of Bax, with the sequels outlined above. In human breast cancer cells, flavopiridol (100–500 n*M*) acted synergistically with epothilone B (20 n*M*) in inducing apoptosis, but only when applied after, and not in advance of, that drug.[85] In a Bcl-2 overexpressing cell line, epothilone B and flavopiridol, alone or in sequence, induced only minor apoptosis, which demonstrates again that Bcl-2 acts as an epothilone antagonist.

Specific farnesyl transferase inhibitors are potent antitumor drugs. The explanation is that they prevent posttranslational farnesylation of certain proteins (e.g., of the p21ras family), which is a prerequisite of their insertion into the cell membrane. With human breast cancer cells, it was shown that those inhibitors (e.g., L-744,822; Merck) increase sensitivity to antimitotic drugs, such as Taxol and epothilone.[86] The synergistic effect was additive. It is speculated that this happens because a farnesylated protein controls the checkpoint of mitosis. Also, human prostate cancer cells, including a line that was rather resistant to L-744,822, became considerably more sensitive to the drug when they were pretreated with epothilones.[52] This was regarded as a promising strategy for therapy of advanced prostate cancer. A broad synergistic effect was seen in colon cancer cells treated with a combination of epothilone D and 5-fluorouracil.[87]

Histone-deacetylase inhibitors, like LAQ824 (Novartis), lead to altered gene expression, with changes in the levels of several important regulatory proteins. As a result they enhance epothilone B–induced apoptosis in human breast cancer cells.[88]

Epothilone B–lactam sensitizes human lung cancer cells to radiation, *in vitro* as well as *in vivo* (athymic nude mice).[89] *In vitro*, the induction of apoptosis by drug and radiation was additive, and a

higher dose of the drug for a shorter exposure time was more efficient than a lower dose for a longer time. The *in vivo* effect is considered to be a result of a combination of mitotic arrest and reoxygenation of the tumor, so that the timing of the irradiation after drug exposure becomes a critical factor.

IV. TOTAL SYNTHESIS AND SEMISYNTHESIS

A. Total Synthesis

The discovery of the epothilones as the first Taxol mimics, and reports of their promising pharmacological properties, triggered enormous worldwide synthetic activities in 1995. With seven stereogenic centers in a 16-membered macrocycle, their total synthesis appeared, though challenging, to be far less difficult than that of Taxol. Thus, even smaller groups entered the field and made epothilone probably the most often synthesized natural product in recent years. Apart from preliminary synthetic studies, more focused work started in late 1995, when the absolute configuration became available from our lab (before publication in July 1996, the absolute configuration of epothilone A and B had been communicated to interested scientists from October 1995 on). First total syntheses of epothilone A and, later, of epothilone B were reported by the groups of Danishefsky, Nicolaou, and Schinzer in 1996 and 1997.[10a] Key bond-forming steps in these early syntheses were Yamaguchi macrolactonization, a highly diastereoselective C6–C7 aldol reaction, and olefin metathesis at C12–C13. Soon after, macroaldolization forming the C2–C3 bond and a novel B-alkyl Suzuki coupling of C11–C12 were introduced by the Danishefsky group. Over the years, these basic approaches have been developed further by the original authors and adopted by many other groups for their own synthetic strategies. Many more alternatives were developed, omitting only a few carbon–carbon bonds not used in the assembly of the macrocycle and the attachment of the thiazole side-chain (Figure 21.6). Thanks to the enormous efforts of many groups, the early synthetic strategies mentioned above proved their value and were adapted to plant-scale syntheses. It is certainly beyond the scope of this review to discuss in detail the more than 20 direct and formal total syntheses published and reviewed already[10a,b,d,f,j–p]; however, a few points on the bond-forming strategies should be made.

In most syntheses, the first strategic goals are, as in biosynthesis, the 12,13-olefins epothilone C and D (occasionally named also dEpoA and dEpoB, **2a**, **2b**), which are epoxidized in a more or less stereoselective manner to epothilone A and B.[125,126] Alternatively, the sensitive epoxide is

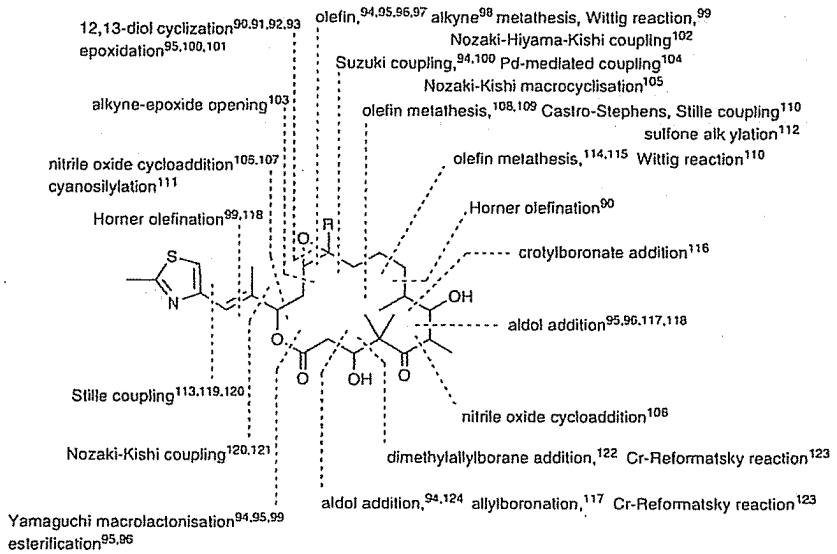


FIGURE 21.6 C,C- and C,O-bond-forming strategies in the total syntheses of epothilones.

introduced at an early stage with high stereoselectivity, and with the necessary precautions, carried through the synthesis by the Mulzer group.[90] Soon after completion of the synthesis of the four major epothilones, A–D, the groups of Danishefsky and Nicolaou and others started to explore structure–activity relationships for cytotoxicity and tubulin binding. Following their own philosophies, either a limited number of selected analogs or large libraries were synthesized via combinatorial chemistry. In this work, all stereocenters were probed for their relevance for biological activity, the alkyl groups on C8[127] and C16[128,129] were removed, or new ones were added at C10[130] and C14.[130,131] Further, the C6[64] and C12 methyl groups were replaced by larger alkyl and modified alkyl groups.[49,115,119,128,132–136] C17–C18 Stille coupling allowed the introduction of a great variety of five- and six-membered heterocyclic side-chains, optionally carrying additional substituents.[113,119,129] Another obvious modification, the ring closure of C27 with the thiazole ring, was first realized by the Novartis group only in 2000 with the synthesis of benzthiazole, benzoxazole, and quinoline analogs.[137] The 12,13-*cis* epoxide was changed to *trans* and, moreover, replaced by a cyclopropane,[120,121,138–141] cyclobutane,[121,140] or aziridin ring.[142]

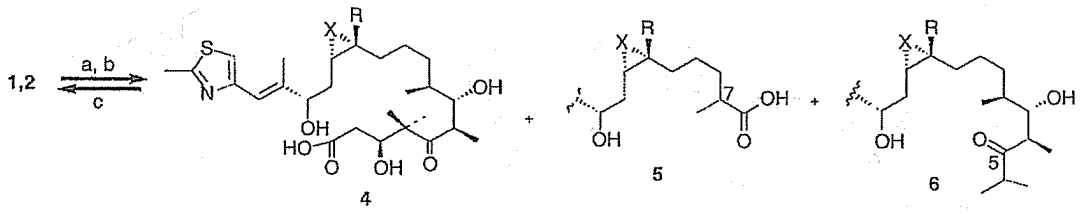
Whereas 12,13-trans olefins and the epoxides derived from them were regularly unwanted by-products in the ring-closing olefin metathesis, Altmann et al. synthesized them in a stereoselective manner to prove their unexpected biological activity,[143] which had been observed earlier by Nicolaou and coworkers.[144] To rigidify the macrocycle and enforce certain conformations, 12,13-benzo,[145] 10,11,12-benzo,[64,146] and 10,11-cyclopropano[108] analogs, and a C23, C24 ethanol bridged analog,[91] were synthesized. Similarly, the C4 geminal dimethyl group was integrated in a cyclopropane ring,[53,54] 11-hydroxy and 11-fluoro substituents were introduced,[105] and oxygen was introduced as isosteric replacement of C10.[147] Epothilones with extra double bonds at C9, C10[101,115b,148,149] and C10, C11,[108] or with a C9, C10 alkyne bond,[150] became available *en route* to epothilone B and D.

Occasionally, natural product building blocks were introduced, such as nerol[123,151] and carene[152] in the northern ring segment. The use of pantolactone[64,153] for the C1–C5 ring segment and malic acid[64] for the C13–C16 fragment provided the C3 and C15 stereocenters. In one and the same synthesis, both northwestern and southeastern ring segments were constructed from glucose by extensive functional group manipulations performed by Potier et al.[154] Chemo-enzymatic methods were applied to establish the desired configuration at the C3, C7, and C15 hydroxyl groups. Thus, resolution was achieved by commercial esterases and lipases,[155,156] by a genetically engineered lipase,[157] or in case of C15, by a catalytic aldolase antibody.[158–160] An aldolase-catalyzed asymmetric synthesis was developed by Wong et al.,[161] providing building blocks with the C7 and C15 stereocenters in enantiomerically pure form. In a polymer-supported synthesis of an epothilone C library, Nicolaou et al. used an olefinic linker to the polymer support, which is cleaved in the final step by participation in an olefin metathesis cyclization.[144,162] In this way, only successfully cyclized molecules are released from the polymer to give immediately pure products. In an alternative approach, Ley et al.[163] synthesized epothilone B, using polymer-supported reagents and scavengers while the growing product was kept in solution. Thus, tedious extraction and purification steps were avoided, making this approach amenable for large-scale synthesis. However, the advantages of the overall process may be doubtful in terms of cost effectiveness when costs for regeneration of the polymer reagents are included.

In summary, during the past eight years of epothilone synthesis, around 160 papers have been published and more than 100 patents filed and, in part, granted. Apart from academic syntheses of natural epothilones and the development of new synthetic methods, around 1000 epothilone analogs may have been synthesized for structure–activity relationships studies. In two cases, epothilone D and epothilone C6–modified epothilone B analogs, large-scale processes were developed, taken to plant-level production by the Danishefsky and Schering groups, and introduced in clinical trials.

B. Semisynthesis

In the course of strain and production optimization at GBF, epothilone A and B became available in larger amounts in 1996 and 1997 and were used in a semisynthesis program in collaboration with

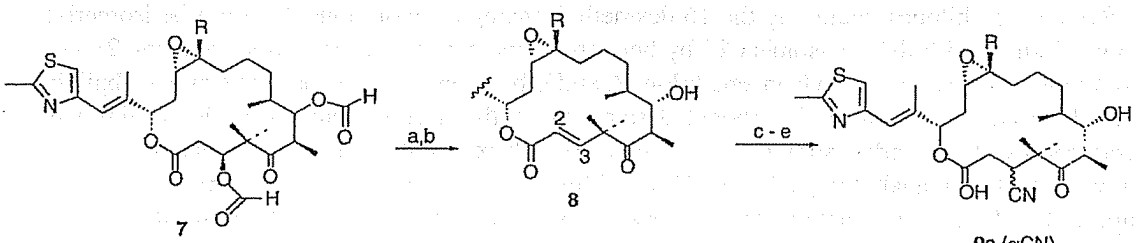


SCHEME 21.2

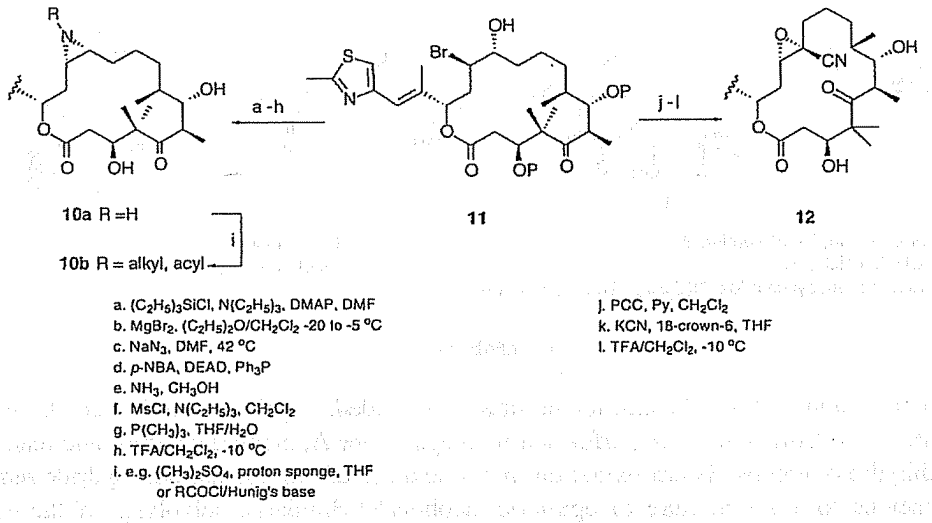
Bristol-Myers Squibb. Later, a block-mutant strain was added, supplying epothilones C and D.[17] Early one-step transformations were performed with epothilone A, including mono- and diacylation of the 3,7-dihydroxy groups, Swern oxidation to 3,5- and 5,7-diketones, and borohydride reduction of the C5 ketone to a 1:1 mixture of epimeric alcohols.[164] Similarly, solvolysis of the epoxide produced mixtures of stereoisomeric diols (cat. H_2SO_4), chlorohydrins (HCl), or rearranged 14-membered lactones (TFA, BF_3OEt_2). Dihydroxylation of epothilone C with OsO_4/NMO gave a 2:1 mixture of epimeric *cis* diols, dimethyl dioxirane, and MCPBA mixtures of natural epothilone A — its β-epoxy isomer — along with other oxidation products.[165,166] 12,13-Dihydro-epothilone C was formed selectively with diimine, whereas catalytic reduction produced complex mixtures because of hydrogenolysis of the allylic ester moiety.[165] Disappointingly, all these derivatives were devoid of significant biological activity.

Hydrolysis of the lactone in epothilones A–D was achieved by LiOH in $MeOH/H_2O$ to give *seco*-acids **4a–d**,[21,167] and depending on reaction time, by retroaldol reactions, the C7 aldehyde and C5 isopropyl-ketone fragments (Scheme 21.2).[21] A very clean cleavage to *seco*-acids was achieved with pig liver esterase.[21,51,167] Interestingly, epothilone A was restored by Yamaguchi macrolactonization of 3,7-nonprotected *seco*-acid **4a** in 25% yield.[21] Although C3 and C7 hydroxyl groups occupy β-positions to carbonyl groups, they are not eliminated under these and other more rigorous conditions. Only the 3,7-diformyl epothilones 7 on treatment with DBU gave in good yield trans-2,3-olefins **8**, which act as Michael acceptors (e.g., for cyanide to give a 1:1 mixture of 3α- and 3β-cyano epothilones **9a** and **9b**; Scheme 21.3).[168]

Another ideal target for structural modification was the 12,13-epoxide. Thus, epothilone A was selectively opened to bromohydrin **11** with $MgBr_2$ in ether, oxidation to the bromoketone, α addition of cyanide followed by ring closure gave the *trans*-like cyanoepoxide **12**.[168] Its structure was proven by x-ray crystallography. Alternatively, the bromohydrin was converted to the azido alcohol, and Mitsunobu inversion and *O*-mesylation set the stage for phosphine reduction and cyclization to aziridin **10a** with the natural α-configuration.[142] Subsequently, the nitrogen was alkylated and acylated with a broad range of residues to **10b** (Scheme 21.4).



SCHEME 21.3



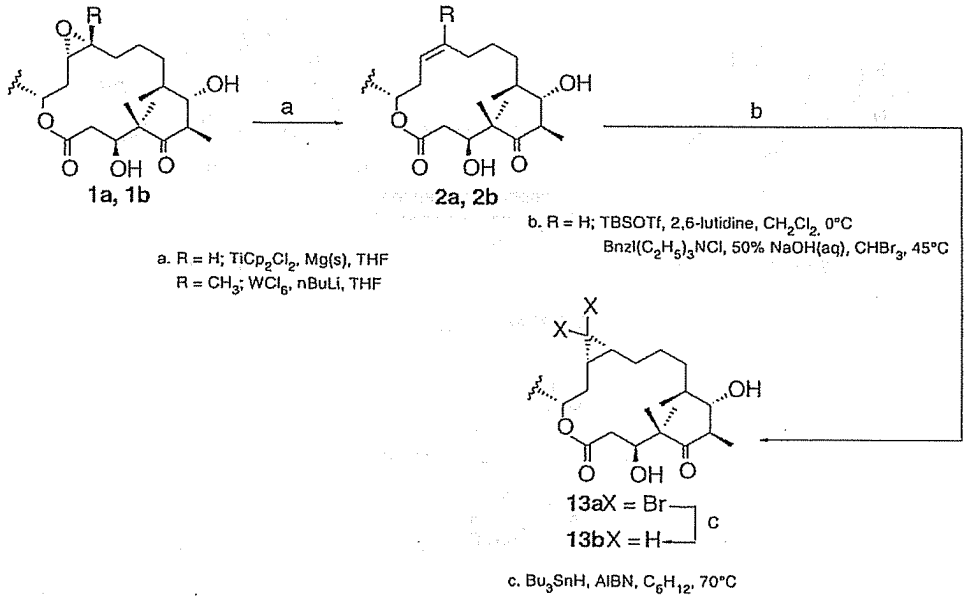
SCHEME 21.4

Whereas epoxidation of the C12, C13 double bond is the final step in epothilone A and B biosynthesis and in many total syntheses, it was desirable to use the rich supply of epothilone A and B from fermentation for the reverse reaction. This was achieved by the BMS group in good yield and retention of (*Z*)-configuration by reduction of unprotected **1a** and **1b** with $TiCp_2Cl_2/Mg$ and WCl_6/n-butyl lithium to epothilone C and D, respectively.[139] Cyclopropanation of the C12, C13 double bond of epothilones C and D turned out to be a tough problem but was eventually achieved in low yield with dibromo- and dichlorocarbene generated under phase transfer conditions to give exclusively α-cyclopropane **13a**.[139] Subsequently, the halogens were removed by tinhydride reduction to **13b** (Scheme 21.5).

Cleavage of the C12, C13 double bond of epothilone C and D by either ozonation or with $OsO_4/Pb(OAc)_4$ or, alternatively, diol formation and periodate cleavage of epothilone A and B gave valuable C12 aldehyde and ketone building blocks ready for, for example, Wittig condensation with synthetic ring segments.[169,170] Inspired by the ring-closing olefin metathesis in the total synthesis of epothilones, the reverse reaction was investigated in presence of an excess of ethylene and second-generation Grubbs's catalysts. The expected *seco*-diene **14** was obtained in good yield and processed further by replacement of the C13–C15 ring segment with a synthetic analog to give **15**. Ring-closing olefin metathesis afforded, for example, the alkyne analog **16** of epothilone C along with the expected stereoisomer at C12/C13 (Scheme 21.6). Subsequently, **16** was epoxidized to the alkyne analog of epothilone A.[171] More direct modifications of the linker group C16,C17 were catalytic hydrogenation, epoxidation followed by reduction to a 16-hydroxyl group,[164] and its elimination to a C16 exomethylene derivative.[21]

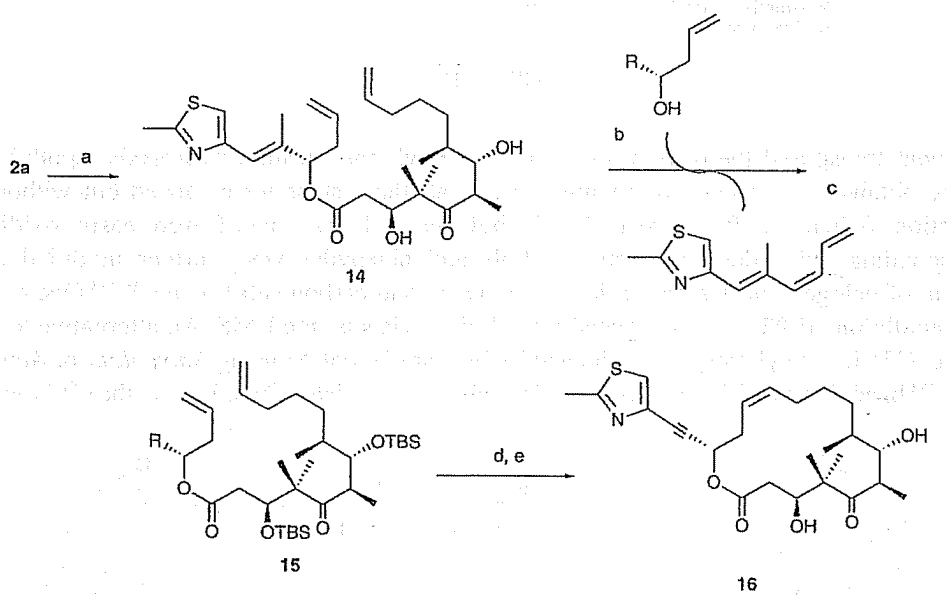
Several epothilones, including the 16-desmethyl analog of epothilone A, could be isomerized to 6:4 mixtures with their *Z*-isomers **17** by benzophenone-sensitized irradiation (Scheme 21.7).[171] C16-ketones **18** were obtained from epothilone A and B by ozonolysis and used as synthetic building blocks.[164] Apart from standard carbonyl derivatives of the acetyl group, new side-chains were introduced by aldol condensation to, for example, **19**, whereas Wittig-like olefinations only worked well with the boron analog $(C_2H_4O_2B)_2CHLi$ yielding vinyl boronic acid **20** (Scheme 21.8). Suzuki coupling of **20** required reactive iodo compounds, such as iodo benzene; otherwise, it was transformed by *N*-iodosuccinimide (NIS) to the corresponding iodovinyl epothilone and introduced in Stille couplings,[166] as described by Nicolaou et al.[119,120]

Initially, modification of the thiazole ring other than by replacement seemed hardly possible in the presence of the sensitive functional groups in the macrocycle. However, with *sec*-butyl and



SCHEME 21.5

t-butyl lithium at low temperatures, deprotonation of C19 and, to a lesser extent, C21 methyl was possible, and after quenching with deuterium chloride or carbon and heteroatom electrophiles, the corresponding derivatives were obtained in moderate to low yields.[172] An unexpected and selective access to C21 derivatives was opened by thiazole *N*-oxidation using *m*-chloroperbenzoic acid (MCPBA). Expectedly, on treatment of the *N*-oxide **21** with acetic anhydride, a Polonovsky-like



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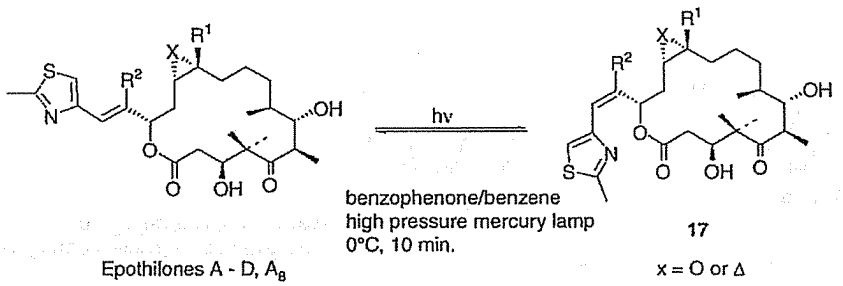

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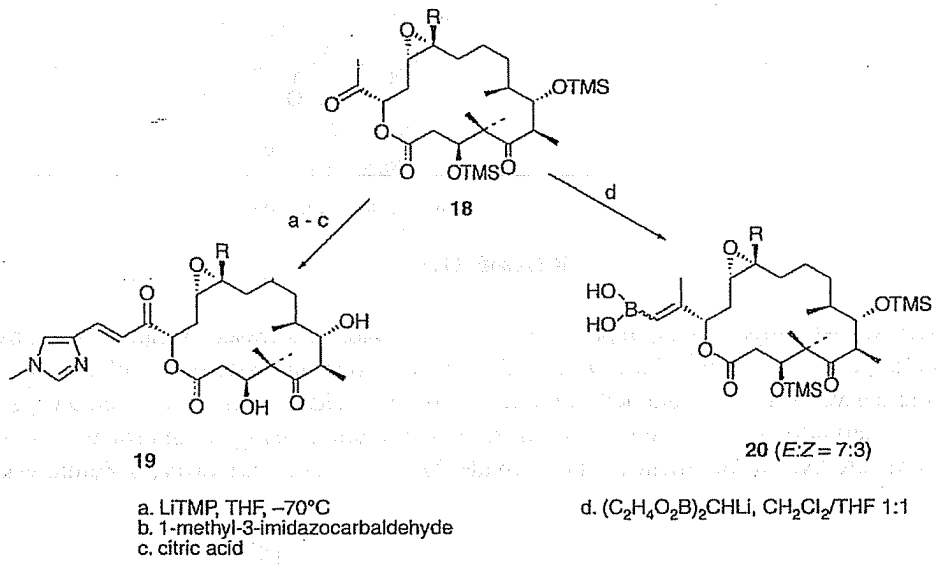
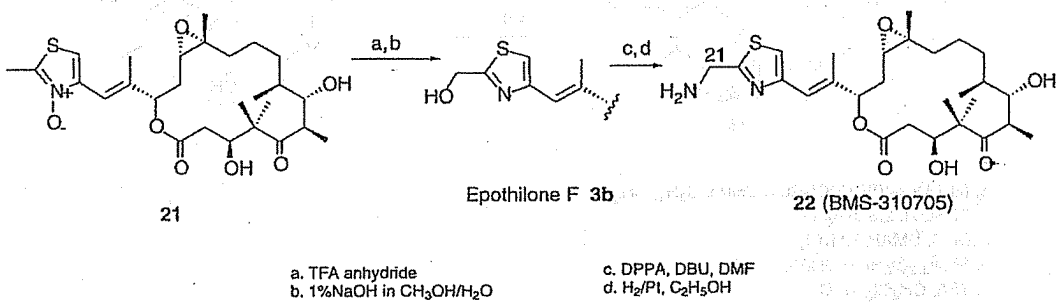


SCHEME 21.8

rearrangement transposed the oxygen to the C21 methyl, and on mild hydrolysis, epothilones E and F were obtained.[50] It should be mentioned that all these steps were carried out without 3,7-OH protection (Scheme 21.9). As benzylic alcohols, epothilones E and F were easily oxidized to the corresponding aldehydes, and both alcohols and aldehydes were further modified by the introduction of halogen and a great variety of N, O, S, and carbon substituents.[50,173] One of these, 21-amino epothilone B **22**, was introduced into clinical trials by the BMS. An alternative to the *N*-oxide route, C21 hydroxylation, was achieved by biotransformation using *Amycolata* or *Actinomyces* strains.[174] In addition to C21, *Am. autotrophica* also hydroxylates C9, C14, and the C12 methyl.[38]



SCHEME 21.9

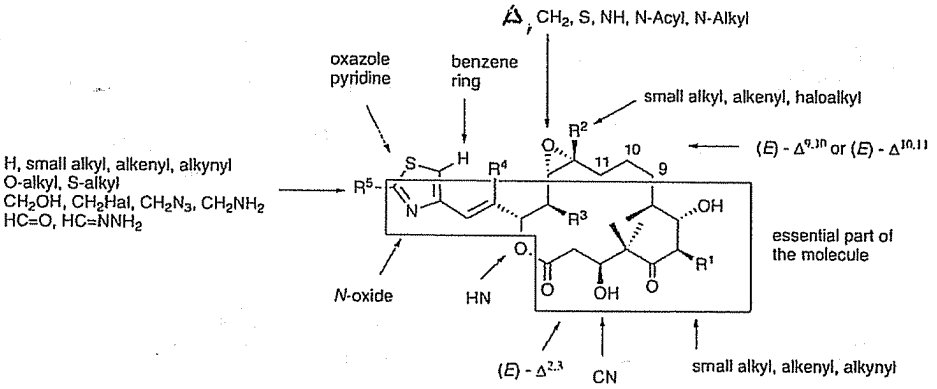


FIGURE 21.7 Structure–activity relationships of epothilone.

slightly, whereas a 14β methyl causes refolding of the corresponding ring segment and loss of activity.[131] The vinyl spacer C16/C15 in the side-chain and the presence and position of the sp^2 nitrogen are of crucial importance for biological activity (Figure 21.8).[49] Shorter spacers or those extended by one atom,[166] (*Z*)-configuration of the double bond,[171] an alkyne spacer,[171] epoxides, or conformationally flexible derivatives with a C16/C17 single bond[164] are inactive. The thiazole may be replaced by oxazole or α-pyridyl (**25**, X = O, **26**), without loss of activity whereas β- and γ-pyridyl analogs are only moderately active or inactive.[4,53,54,64,119]

Likewise, thiazoles linked by their C2 or C5 position are inactive.[113] Integration of the C16, C17 spacer in a benzene ring to give benzothiazole, benzoxazole (**28**), and quinoline (**27**) analogs confers high biological activity.[64,137] In contrast, introduction of any substituent at the thiazole C19 (**29**) or alkylation of the nitrogen (**30**) abolishes activity.[172] The C20 methyl on the thiazole may be replaced by hydrogen, small alkyl, alkenyl, or alkynyl groups[173] or heteroatom substituents with retention of high activity.[141,178] In general, three-atom (except hydrogen) residues are still highly to moderately active, and bigger ones are weakly active or inactive.[173] The methyl group C21 may be modified by a variety of halogen, oxygen, nitrogen, and sulfur substituents or oxidized to the aldehyde and further derivatized without loss of activity, provided the space demand of the entire residue does not exceed three to four atoms.[173] The thiazole-2-carboxylic acid derivative is inactive.[173]

Another part of great structural flexibility is the C12, C13 epoxide. Cyclopropane,[120,139,140] cyclobutane,[140] episulfide,[21,177] and aziridin analogs including *N*-alkyl or *N*-acyl modifications[142] are highly active, whereas the corresponding olefins, epothilone C and D, are moderately active.[97,113,179]

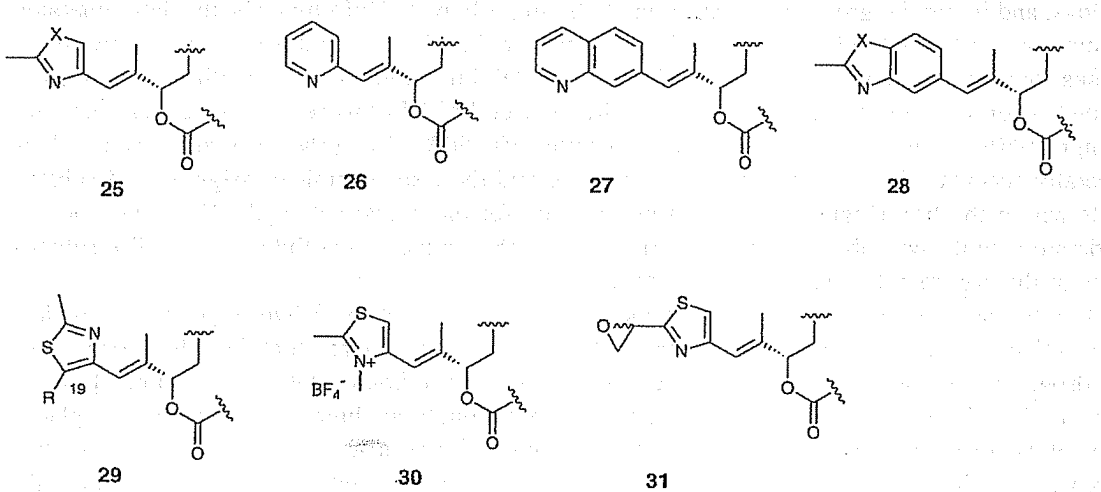
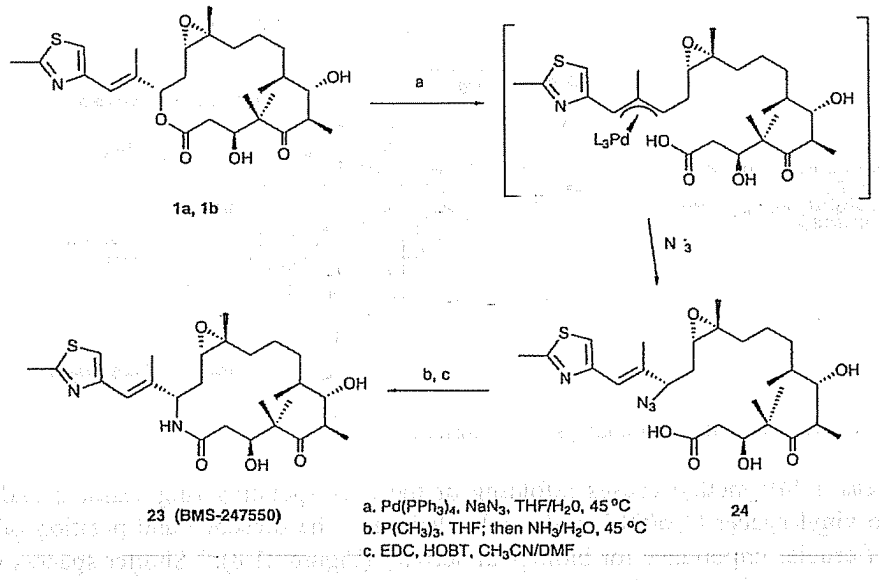


FIGURE 21.8 Side-chain modified epothilones.



SCHEME 21.10

Although obvious, isosteric replacements of the carboxyl group were not covered in the earlier total syntheses but were first realized by semisynthesis. In a very short and elegant reaction sequence, Vite and coworkers at BMS transformed epothilone B lactone to the lactam. Using palladium chemistry, the allylic lactone was opened and the C15 oxygen replaced by azide with overall retention of configuration. Reduction of the azide to an amine, and cyclization by standard peptide chemistry, produced the lactam in good yield (Scheme 21.10).[51] Remarkably, the whole sequence did not require 3,7-OH protection and could be performed as a one-pot reaction. Because of its favorable cytotoxic and pharmacological profile, epothilone B–lactam **23** (BMS-247550, Ixabepilone) was introduced into clinical trials in 2000.[10q]

V. STRUCTURE–ACTIVITY RELATIONSHIPS

Investigation of structure–activity relationships started very early at GBF, with more than 39 natural epothilones isolated from large-scale fermentations and simple one-step semisynthetic derivatives. Shortly after, the Danishefsky and Nicolaou groups published biological data for a great variety of analogs, and industrial groups at Novartis and Schering followed. Unfortunately, the data generated for cytotoxicity/growth inhibition by different groups are hardly comparable on a quantitative basis because of the broad range of sensitivity of the various cell lines used. *In vitro* tubulin polymerization depends even more on the experimental conditions used.[138,175] In this review, we classify activity as high (>50% of the parent compound), moderate (5%–50%), low (1%–5%), and inactive. It is generally accepted that a 16-membered macrocycle and the stereochemical assignment of substituents within the box (Figure 21.7) are a prerequisite for biological activity.[113] However, good to moderate activity was observed with certain 17- and 18-membered epothilones.[113,176] The substituents in the box may be modified to a certain extent.

Epothilones with an (*E*)-2,3-double bond from elimination of the 3-hydroxyl are still moderately active, whereas 3β-cyano analogs are highly active.[168] This means that the 3-hydroxyl is not a hydrogen bridge donor but, rather, an acceptor in the tubulin bound state of epothilone. Both 4-desmethylepothilones A_1 and A_2[4] and 16-desmethylepothilone[129] are highly active, whereas replacement of 8-Me by hydrogen drastically reduces activity.[127] Extension of the C6 methyl to ethyl increases activity fourfold compared with epothilone B, and allyl and propyl derivatives are only slightly less active than the parent compound.[64] An extra methyl in the 14α position reduces activity

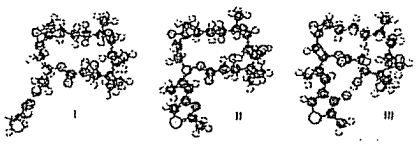


FIGURE 21.9 **(See color insert following page 304.)** Crystal structures of epothilone B (**1b**) from dichloromethane/petroleum ether (I), methanol/water (II), and epothilone A-*N*-oxide (III).

Remarkably, the latter are almost as active as their epoxides in the *in vitro* tubulin assay,[162,179] a fact that rules out an early hypothesis of covalent binding to tubulin protein via epoxide opening. Whereas β-epoxides and their analogs are entirely inactive, inversion of the configuration only at C12, corresponding to a C12, C13-*trans* configuration, retains activity.[162] The same applies for the introduction of *trans* double bonds in the C9, C10,[115,148,149] and C10, C11 position.[4,108,109] The C12 methyl is extremely important for high activity of the epothilone B series. However, it may be replaced with retention of activity by small alkyl, alkenyl, and alkynyl groups or modified by introduction of halogen and oxygen substituents.[49,132,136] Interestingly, the fluorescent epothilone D analog with a large *m*-dimethylaminobenzoyl residue is still about as active as Taxol.[180] Last but not least, replacement of the lactone group of epothilone B, D, and F by a lactam reduces cytotoxic activity and tubulin binding slightly, while other properties such as the therapeutic window or p.o. efficacy are improved.[51,57] Remarkably, lactam analogs obtained by total synthesis rather than semisynthesis seem to be less active.[48,181]

Studies on the solution conformation of epothilone A started even before the absolute configuration was published. Thus, Georg's group predicted[182] the relative configuration and conformation of epothilone A on the basis of nuclear magnetic resonance measurements and molecular dynamics calculations. One of the proposed structures showed the correct relative configuration for the independent western and eastern ring segments. We found NOEs and coupling constants of epothilone B in DMSO/D_2O solution in good agreement with a conformation similar to that in the crystal.[3] Only the thiazole side-chain is apparently rotating around both its single bonds. In a detailed conformational analysis, Taylor and Zajicek[183] added a second minor conformer, designated B, with the 3-OH rotated from an axial to an equatorial position, accompanied by rotations in the C6/C7 segment. Interestingly, an equatorial 3-OH is also found in the crystal structure of epothilone B-*N*-oxide, where it is stabilized by a hydrogen bridge to the *N*-oxide, while the conformation around C6-C8 is not influenced.[50] Apparently, a weak intramolecular hydrogen bond of the 3-OH to the thiazole nitrogen is also able to stabilize this 3-OH_{eq} conformation in solution, as we found in (21*R*,*S*)-diastereomeric C21-substituted epothilone A and B derivatives.[173] Thus, in the 20-epoxyethyl derivative **31** (Figure 21.8), the 3-OH nuclear magnetic resonance signal appears duplicated, and a strong NOE is observed between 17H and 3H. The latter indicates a C16–C19 *syn*-periplanar conformation as it is observed in the crystal structure of epothilone B from an aqueous solvent (Figure 21.9).

Most remarkably, the same conformational changes were recently proposed for the tubulin-bound state of epothilone A from sophisticated nuclear magnetic resonance measurements (transferred NOEs and transferred cross-correlation rates) by the Griesinger group.[184,185] However, the unusual experimental conditions employed and the lack of appropriate controls cast some doubts on the relevance of these findings.[186] In fact, changes may have been induced by the aqueous solvent of high ionic strength rather than specific binding to tubulin. Earlier results on the bioactive conformation of epothilones based on computational methods were published in a series of papers in 1999 and 2000. Inspired by some intriguing structural similarities of epothilone and Taxol and by the fact that Taxol is replaced from its binding site by epothilone, common pharmacophore models were postulated and corroborated by mapping of cross-resistance to certain amino acid replacements in β-tubulin.[187–189] Recently, a tubulin point mutation outside the Taxol binding site was also observed that confers high resistance to both Taxol and epothilone.[73] Even though the

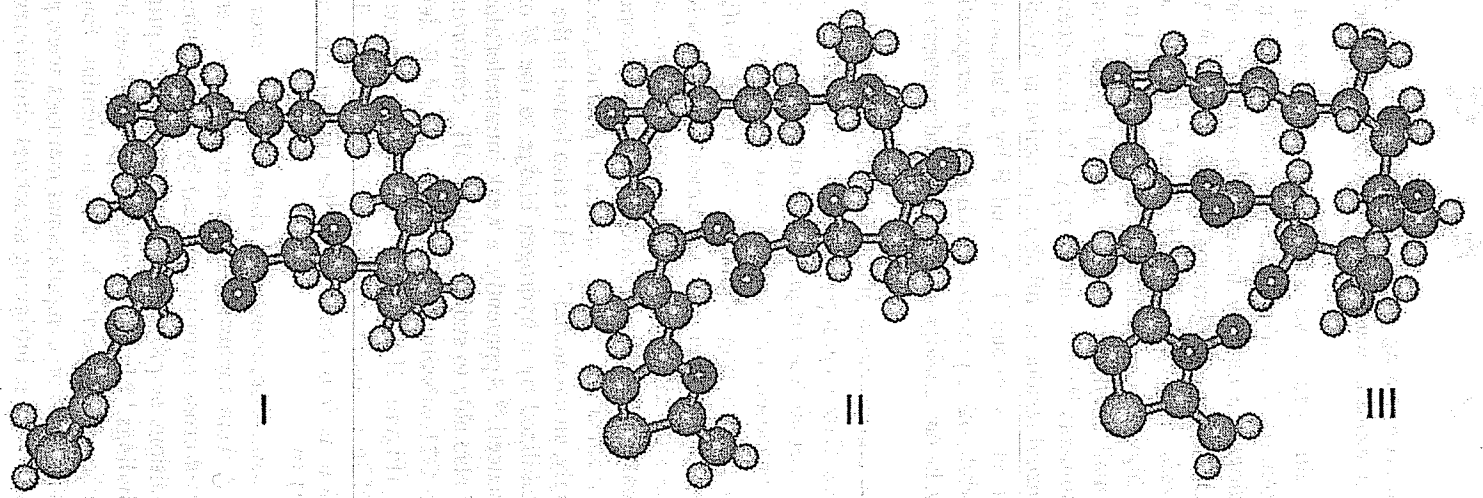


Fig. 21.9

binding models proposed are contradictory, there are some similarities. Thus, after discovering that the C13 side-chain of Taxol is not absolutely necessary for tubulin binding, Kingston and Horwitz[189] proposed that the C2 benzoyl residue of Taxol and the thiazole side-chain of epothilone occupy the same region of the protein. The same applies for the (less-favored) binding mode I proposed by Giannakakou et al.[70] Surprisingly, little use is made of conformationally restrained epothilone analogs and the highly active C12,C13 *trans* isomers in these modeling studies. From replacement of the thiazole by a pyridine and modifications of the C16–C17 linker we know that the nitrogen has to be exactly positioned in the binding site serving presumably as hydrogen bond acceptor. The bioactive conformation of the thiazole ring is clearly C16–C19 *syn*-periplaner from the fact that no substituents are tolerated at C19[166,172] and from the high activity of the conformationally *syn*-locked benzothiazole and quinoline analogs.[64,137] Interestingly, the opposite, a C16–C19 anti-periplaner conformation of epothilone D and B, is adopted in the crystal structure of the complex with cytochrome P450epok,[45] the biosynthetic enzyme that introduces the epoxide. Clearly, major advances of our knowledge of the epothilone–tubulin binding have to come from crystallographic and photoaffinity labeling studies, as was the case with the taxoids.

VI. PRECLINICAL STUDIES

Epothilone effects, as far as they are published, have been determined mostly in athymic nude mice but occasionally also in severe combined immunodeficiency (SCID) mice, nude rats, and beagle dogs, in most cases with xenografts of human cancer cells. The data are even more heterogeneous than those with cell cultures because of differences of the method and schedule of application and because of the kinds of cancer cells, epothilone analogs, and experimental animals used. The studies established, nevertheless, that epothilones act *in vivo*; that they inhibit human cancers, often resulting in cures of the animals; and that they are superior to Taxol and other cytostatic drugs in many cases, especially with MDR tumors. Severe combined immunodeficiency mice tolerate epothilone B well at a dose of 0.3 mg/kg; at 1 mg/kg, weight loss is observed; and at 3 mg/kg the animals show severe toxic effects (once per week, subcutaneously, vehicle 30% PEG + 70% 0.9% NaCl solution).[84]

In a preliminary study on nude mice, a dose of 0.6 mg/kg epothilone B, daily for 4 days, intraperitoneally, killed all animals, whereas all survived treatment with 25 mg/kg, daily for 5 days, of epothilone D.[46] It was suggested that the epoxy group at C12/C13 may be responsible for high *in vivo* toxicity.[55] Apart from leukopenia (43% decrease of lymphocytes), no hematological or other pathological changes in various organs were seen with either drug.[46] Toxicity of epothilone B could be mitigated by an altered application schedule: at 0.6 mg/kg, 5 times every second day, intraperitoneally, in DMSO, only three of seven mice died, and with the same dosage, but intravenously, all animals survived. Although epothilone D treatment resulted in appreciable regression and even cures of human mammary and ovarian carcinoma xenografts, epothilone B had too narrow a therapeutic window for good antitumor effects. The mode of application was later optimized intravenously by infusion over 6 h rather than by injection, using the vehicle cremophore-ethanol.[23a,56]

The benefits of epothilones are most impressive on MDR tumors. Thus, in nude mice epothilone D was >35,000-fold more active than Taxol on MDR human adenocarcinoma. It also was fully curative for human T-cell leukemia.[23a] A comparison of several epothilones, Taxol, and other cytostatic drugs showed superior performance of epothilone D and its C21–OH analog (d Epothione F) in many cases.[23a,b] Epothilone D (30 mg/kg, intravenously) was better than Taxol in controlling xenografts in nude mice of human solid lung, mammary, and one of two ovarian adenocarcinomas, and in particular of MDR sublines. It was inferior to Taxol, however, with colon, colon adeno, prostate adeno, and one of the ovarian adenocarcinoma lines. Complete remission was only achieved with MX-1 mammary carcinoma (as well by Taxol). Epothilone D was superior to Taxol, with complete remission, in four human leukemias, including MDR lines, and was inferior to Taxol only in the HL-60 line. Epothilone B and epothilone B–lactam were much less successful because of their narrow therapeutic windows (but see below). The same study showed that resistance to

epothilone D developed only very slowly and to low levels (twofold in 21 months).[56] In mouse plasma, epothilone D was quickly inactivated ($t_{1/2}$ = 20 min), probably because of esterase activity, but was much more stable in human plasma ($t_{1/2}$ = >3 h).[56] Beagle dogs fully tolerated a single dose of 2–6 mg/kg, intravenously (40–120 mg/m^2); they developed diarrhea and leukopenia and lost weight at 12 mg/kg (240 mg/m^2); and 20 mg/kg (400 mg/m^2) produced severe toxic effects (e.g., necrosis of the intestinal mucosa, cell decay in the bone marrow) and was lethal.[56] At 6 mg/kg, $t_{1/2}$ was >5 h, and plasma concentrations after 24 h were 0.045 μg/mL (0.092 μ*M*), which is 10 times the IC_{50} (0.0095 μ*M*) for leukemia cells.[56]

Recently, more effective epothilone analogs have been tested.[136] Introduction of a C9, C10 double bond in epothilone D makes the compound 10 times more active on MX-1 mammary carcinoma xenografts in nude mice, but also more toxic (with a maximum tolerated dose [MTD] of 3 mg/kg, versus that of epothilone D [30 mg/kg]). Also, although the compound may lead to a complete remission of tumors, they often later come back. Performance could be much improved by further replacing the CH_3 on C12 by CF_3, which bestows higher stability ($t_{1/2}$ = 212 min in mouse plasma, 10.5 h in human plasma; epothilone D, 46 min) and bioavailability (solubility in water: 20 μg/mL; epothilone D: 9.4 μg/mL). Thus, for example, with xenografts of human slow-growing lung carcinoma, A549, 25 mg/kg, 6 times every second day twice 8 days apart, resulted in 99.5% remission and, after two more doses, in complete elimination (epothilone D could not eradicate the tumor). Growth of a Taxol-resistant subline was completely stopped, and tumor size reduced by 24% at 20 mg/kg, 7 times every second day.[136]

Another novel compound, semisynthetic C-21 thiomethyl epothilone B (ABJ879), has just been proposed by Novartis for a phase I clinical study.[190] It shows about the same tubulin-polymerizing activity as Taxol but is clearly more potent in blocking cell proliferation in cultures (average IC_{50} for seven human carcinomas: 0.09 n*M* vs. 4.7 n*M* of Taxol). The drug is fully active on MDR1 cells and lines with resistance resulting from β-tubulin mutations. In nude mice, single-dose treatment (2–3 mg/kg, intravenously, 6–7 d after implantation of the tumor) produces transient but long-lasting remission (25%–95%). Positive effects were seen with slow-growing lung adenocarcinoma H-596, colon tumor HT-29, and large-cell lung tumor H-460. A preliminary pharmacokinetics–pharmacodynamics drug-response model has also been presented.[191] Doses of 1.5 and 1.8 mg/kg, every second week, were acceptable, but 1.5 and 2 mg/kg, once a week, caused intolerable weight losses. The drug penetrates tissues, where its level becomes 10 times higher than in plasma, and is retained there for a long time, so that plasma concentrations are not representative of tissue exposure — a fact that may be true also for other epothilone analogs.

The controversy about the efficacy of epothilone B–lactam (BMS-247550) has been fueled by decidedly positive results reported by BMS scientists.[57] In addition to various established tumor cell lines, three primary xenografts from biopsies were tested. A human ovarian carcinoma, Pat-7, from a patient who no longer responded to Taxol and other cytostatic drugs was highly responsive to epothilone B–lactam in nude mice at the optimal dose of 4.8–6.3 mg/kg, intravenously, 5 times every second day (the optimal dose of Taxol is 24–36 mg/kg). The log cell kill (LCK) was 2.9 (Taxol, 0.8). The same tumor in nude rats at the optimal dose of 3 mg/kg, intravenously, two times eight days apart, could be eradicated in four of six animals (LCK > 5; Taxol, LCK = 2.2, no cures [0/6]). Good LCK values and growth delay were also seen in nude mice with Taxol-resistant human ovarian and colon carcinomas. Also, in several other instances of Taxol-sensitive tumors in nude mice, the animals could be cured, and high LCK values (>6.3) were seen, even when the compound was not better than Taxol. Furthermore, epothilone B–lactam gave excellent results when administered orally. At the MTD (60–80 mg/kg, 5 times every second day), epothilone B–lactam was highly active on the Pat-7 xenograft (LCK = 3.1, growth delay of 32.8 d; in comparison, Taxol, intravenously, LCK = 1.3, growth delay of 9.8 d, orally inactive). HCT116 colon carcinoma was eradicated in all eight mice treated with 90 mg/kg, 5 times every second day (equivalent to the best intravenous regimen). In contrast to epothilone A and B, epothilone B–lactam is completely stable in rodent and human plasma and in liver microsomal preparations and shows clearly reduced toxicity.[57]

Epothilone D, too, was reported to be active in rats and beagle dogs after oral administration.[192,193] The drug was well tolerated, and >50% (dogs) and 10%–20% (rats) was found in the bloodstream, with half-lives of 9–11 h (dogs) and 6 h (rats), respectively. A study by the Sloan-Kettering Institute for Cancer Research yielded far fewer positive results with respect to epothilone β lactam in comparison to their own epothilone D.[55] At 6 mg/kg, 4 times every day, intravenous infusion, MX-1 mammary tumor xenografts in nude mice were somewhat inhibited in growth, but no remission was seen, and the effect was not much improved by treatment with doses near the MTD (9 mg/kg). Similar results were obtained with K562 leukemia, which could be cured, however, when the animals subsequently were treated with C-21-OH epothilone D (30 mg/kg).

A study, to date only *in vitro*, indicates that the dosing regime could have a massive influence on tumor angiogenesis and metastasis.[194] Human vascular endothelial cell lines were more efficiently killed by a "metronomic" administration of cytostatic drugs, including epothilone B; that is, by extended (144 h) application of low (10–100 p*M*) concentrations rather than high doses for a short time (24 h). IC_{50} values were thus reduced to 25–140 p*M*, although those for tumor cells and fibroblasts remained high (550 p*M* to >1 n*M*), so that this strategy appears to selectively affect endothelial cells.

Not much has been published about the metabolic fate of epothilones in animals. Degradation of epothilone B (10 mg/kg, intravenously) in the liver of nude mice was studied by capillary high performance liquid chromatography combined with mass spectrometry.[195] One hour after administration, three metabolites could be recovered and identified guided by a key fragment at m/z166, an acylium ion derived from the very stable thiazole side-chain. The following degradation pathway was reconstructed: opening of the epoxide, opening of the macrolactone ring to the hydroxycarboxylic acid, reduction of the C5-keto group, and reaction of the C5-hydroxy group with the carboxylic acid group to give a six-membered lactone ring or, after conjugation with taurine, to yield a six-membered lactam ring at the end of the chain.

VII. CLINICAL APPLICATIONS

Five epothilone analogs have been introduced into clinical studies (Figure 21.10). Epothilone B (**1b**, EPO906, Patupilone, Novartis) started phase I, beginning early in 1999, followed by phase II.[10n,196] Epothilone B lactam (**23**, BMS-247550, Bristol-Myers Squibb) started phase I beginning October 1999 and completed September 2000; phase I in children started in April 2002; phase II trials have been operative since February 2001, and in 2002, there were 15 National Cancer Institute–sponsored phase II studies; it is presently in phase III.[10q,197] Epothilone B C21-amine (**22**, BMS-310705) is in phase I (2002),[10s,198] and epothilone D (**2b**, KOS-862, Kosan) is in phase I.[136,192,193] Also in phase I is a synthetic epothilone of undisclosed structure, ZK-EPO (**32**, Schering). Epothilone B C2-thiomethyl (**33**, ABJ879, Novartis) is a recent candidate for a phase I study.[190,191] Although most data are only published as abstracts, several excellent reviews summarize the results,[10n,q,s,196,199] so that details need not be repeated here. The epothilone B lactam studies are by far the best documented ones.

The epothilones have been tested in patients with many different carcinomas, mostly solid tumors, including MDR cases and others that are difficult to treat (e.g., cervical adenocarcinomas of the squamous type).[200] The responses have varied between complete and partial remission, tumor stabilization, reduction of CA 125 levels (cancer antigen found at elevated levels in the blood of many cancer patients, especially those with ovarian cancer), and continued growth. The individual epothilone analogs appear to differ in the MTD, the dose-limiting toxicity (DLT), the side effects, half-life in the patient, and efficacy. It may still be too early to assess the quality of the drugs conclusively.

For epothilone B lactam, the MTD was estimated to be 6 mg/m^2, intravenously, over 1 h, daily for 5 days every 3 wk.[201] At 8 mg/m^2 dose-limiting neutropenia was seen, but many patients tolerated higher doses when the drug was applied in combination with filgrastim (granulocyte colony-stimulating

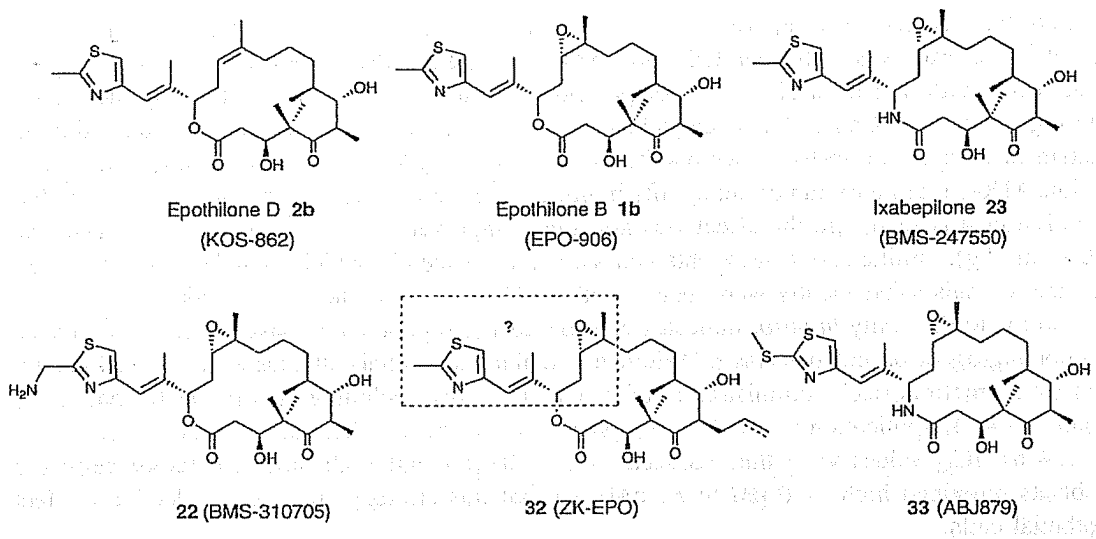


FIGURE 21.10 Epothilones in clinical trials.

factor, which mitigates myelosuppression and neutropenia). Other side effects were peripheral neuropathy, fatigue, nausea, stomatitis, myalgia, and arthralgia, usually in mild forms.[10q,201,202] DLT was neutropenia. Two patients died of sepsis, probably as a consequence of neutropenia. The acceptable weekly dose may be 20–30 mg/m^2. The drug was quickly absorbed by the tissue, where its half-life was 24–48 h, whereas $t_{1/2}$ in the plasma was only 16.8 h. This was also supported by a study that analyzed effects in peripheral blood mononuclear cells in patients.[197] It showed that MTs formed bundles 1 h after application (at the already toxic dose of 50 mg/m^2, intravenously, over 1 h, once a week every 3 wk, in 70% of cells; at 40 mg/m^2 in 63%), but after 24 h, the effect was much reduced (to 25% and 16%–23%, respectively). Cytotoxic effects followed with a time delay on MT bundling but were expressed within 24 h: Cleavage of poly(ADP-ribose)polymerase, indicating cell death (apoptosis and necrosis), was at base level after 1 h (3%) but rose to 28% of cells after 24 h. For treatment of progressive metastatic prostate cancer, a dose of 35 mg/m^2, intravenously, over 3 h, once every 3 weeks (five cycles) in combination with estramustine phosphate (an estradiol analog without affinity to the estradiol receptor) is recommended.[202] Positive responses and decline in prostate-specific antigen levels were seen in 40%–60% of patients. It appears that in aqueous solution the epoxide ring of epothilone B lactam opens to give the inactive diol.[198]

For epothilone B,[199] the MTD is 6 mg/m^2, intravenously, once every 3 weeks, or 2.5 mg/m^2, intravenously, once every week. The DLT was diarrhea. Other toxic effects (neuropathy, nausea) were mild to moderate, and significant myelosuppression, mucositis, and alopecia were not registered. Blood distribution was multiphasic. An initial half-life of 7 min was followed by a terminal half-life of 4–5 d, which indicates fast absorption by the tissue. There was no excretion of unaltered compound via the kidneys (<0.1%).

For epothilone D, reports on clinical performance still are very preliminary.[193] Five patients were treated with doses of 9 and 18 mg/m^2 (intravenously, once every 3 weeks), at which no DLT was seen (mild to moderate emesis and anemia). The elimination half-life of the drug was 5–10 h, and plasma concentrations declined rapidly after infusion in a biphasic pattern.

NOTE ADDED IN PROOF

Since the completion of this article in July 2004 a considerable number of papers on biology and chemistry of epothilones were published. Among the most important ones is that by Nettles et al. on the structure of the epothilone A/tubulin complex.[203] As with taxol, the structure was solved

from two-dimensional crystals by electron crystallography and molecular modelling. Expectedly, epothilone occupies part of the taxol binding site, however, its conformation and contacts to the protein are profoundly different from previous proposals.[204]

ACKNOWLEDGMENTS

We thank all former group members at the GBF for their contributions and the colleagues at Bristol-Meyers Squibb for a fruitful collaboration in the epothilone project. We further thank Mrs. Monica Kirchner for taking care for literature references and typing the manuscript. Financial support from Bristol-Myers Squibb and the Fonds der Chemischen Industrie is gratefully acknowledged.

REFERENCES

1. Höfle, G., Biologically active secondary metabolites from myxobacteria — isolation and structure elucidation, in *Scientific Annual Report of the GBF*, Walsdorff, H.-J., Ed., Gesellschaft für Biotechnologische Forschung, Braunschweig, 1991, 65-68.
2. Gerth, K. et al., Epothilones A and B: Antifungal and cytotoxic compounds from *Sorangium cellulosum* (Myxobacteria), *J. Antibiot.*, 49, 560, 1996.
3. Höfle, G. et al., Epothilone A and B — Novel 16-membered macrolides with cytotoxic activity: isolation, crystal structure, and conformation in solution, *Angew. Chem. Int. Ed.*, 35, 1567, 1996.
4. Hardt, I.H. et al., New natural epothilones from *Sorangium cellulosum*, strains So ce90/B2 and So ce90/D13: isolation, structure elucidation, and SAR studies, *J. Nat. Prod.*, 64, 847, 2001.
5. Höfle, G. et al., German Patent 4138042, filed Nov. 11, 1991, granted Oct. 14, 1993; World Patent 9310121 filed Nov. 19, 1992.
6. See, e.g., Reichenbach, H. and Höfle, G., Biologically active secondary metabolites from myxobacteria, *Biotech. Adv.*, 11, 219, 1993; Höfle, G. and Reichenbach, H., The biosynthetic potential of the myxobacteria, in *Sekundärmetabolismus bei Mikroorganismen*, Kuhn, W. and Fiedler, H.-P., Eds., Attempto, Tübingen, 1995, 61-78; Reichenbach, H. and Höfle, G., Myxobacteria as producers of secondary metabolites, in *Drug Discovery from Nature*, Grabley, S. and Thiericke, R., Eds., Springer, Berlin, 1999, 149-179.
7. Lavelle, F., What's new about new tubulin/microtubules binding agents? *Exp. Opin. Invest. Drugs*, 4, 771, 1995.
8. Bollag, D.M. et al., Epothilones, a new class of microtubule-stabilizing agents with a Taxol-like mechanism of action, *Cancer Res.*, 55, 2325, 1995.
9. Gerth, K. et al., Myxobacteria, proficient producers of novel natural products with various biological activities past and future biotechnological aspects with the focus on the genus *Sorangium*, *J. Biotechnol.*, 106, 233, 2003.
10. a) Nicolaou, K.C., Roschangar, F., and Vourloumis, D., Chemical biology of epothilones, *Angew. Chem. Int. Ed. Engl.* 37, 2014, 1998; b) Mulzer, J., Martin, H.J., and Berger, M., Progress in the synthesis of chiral heterocyclic natural products: epothilone B and tartrolon B, *J. Heterocyclic Chem.*, 36, 1421, 1999; c) G. Höfle, Epothilone — a natural product on the road to becoming a medicine, in *Scientific Annual Report of the GBF*, Jonas, R., Ed., 1999/2000, 21; d) Altmann, K.-H. et al., Epothilones and their analogs — potential new weapons in the fight against cancer, *Chimia*, 54, 612, 2000; e) Altmann, K.-H., Wartmann, M., and O'Reilly, T., Epothilones and related structures — a new class of microtubule inhibitors with potent *in vivo* antitumor activity, *Biochim. Biophys. Acta*, 1470, M79, 2000; f) Mulzer, J., Epothilone B and its derivatives as novel antitumor drugs: total and partial synthesis and biological evaluation, *Monatshefte für Chemie*, 131, 205, 2000; g) Winssinger, N. and Nicolaou, K.C., Epothilones and sarcodictyns: from combinatorial libraries to designed analogs, ACS Symposium Series 796, *Anticancer Agents, Frontiers in Cancer Therapy*, Ojima, I., et al., Eds., American Chemical Society, 2001, 148; h) Altmann, K.-H., Microtubule-stabilizing agents: a growing class of important anticancer drugs, *Curr. Opin. Chem. Biol.*, 5, 424, 2001; i) Stachel, S.J., Biswas, K., and Danishefsky, S.J., The epothilones, eleutherobins, and related types of molecules, *Curr. Pharm. Design* 7, 1277, 2001; j) Nicolaou, K.C., Ritzén, A., and Namoto, K., Recent development in the

chemistry, biology and medicine of the epothilones, *Chem. Commun.*, 1523, 2001; k) Flörsheimer, A. and Altmann, K.-H., Epothilones and their analogs — a new class of promising microtubule inhibitors, *Expert Opin. Ther. Patents*, 11, 951, 2001; m) Wartmann, M. and Altmann, K.-H., The biology and medicinal chemistry of epothilones, *Curr. Med. Chem. AntiCancer Agents*, 2, 123, 2002; n) Borzilleri, R.M. and Vite, G.D., Epothilones: new tubulin polymerization agents in preclinical and clinical development, *Drugs Future*, 27, 1149, 2002; o) Altmann, K.-H., Epothilone B and its analogs — a new family of anticancer agents, *Med. Chem.*, 3, 149, 2003; p) Mulzer, J. and Martin, H.J., *Chem. Rec.*, 3, 258, 2004; q) Lin, N., Brakora, K., and Seiden, M., BMS-247550 Bristol-Myers Squibb/GBF, *Curr. Opin. Investig. Drugs*, 4, 746, 2003; r) Rivkin, A. et al., Application of ring-closing metathesis reactions in the synthesis of epothilones, *J. Nat. Prod.*, 67, 139, 2004; s) Goodin, S., Kane, M.P., and Rubin, E.H., Epothilones: mechanism of action and biological activity, *J. Clin, Oncol.*, 10, 2015, 2004.

11. Reichenbach, H., Order Myxococcales, in *Bergey's Manual of Systematic Bacteriology*, 2nd edition, Garrity, G., Ed., Springer, New York, ~~2004.~~ 2005,
12. *Myxobacteria II*, Dworkin, M. and Kaiser, D., Eds., American Society for Microbiology, Washington, DC, 1993, 1-404
13. Höfle, G. et al., (GBF) German Patent 4211056, 1993.
14. K. Gerth, personal communication.
15. Gerth, K. et al., Studies on the biosynthesis of epothilones: hydroxylation of epo A and B to epothilones E and F, *J. Antibiot.*, 55, 41, 2002.
16. Gerth, K. et al., Studies on the biosynthesis of epothilones: The biosynthetic origin of the carbon skeleton, *J. Antibiot.*, 53, 1373, 2000.
17. Gerth, K. et al., Studies on the biosynthesis of epothilones: the PKS and epothilone C/D monooxygenase, *J. Antibiot.*, 54, 144, 2001.
18. Reichenbach, H. et al., (GBF) German Patent 9647580 (1996).
19. Hofmann, H. et al., (Novartis) World Patent 9942602 (1999).
20. Hecht, H.-J. and Höfle, G., unpublished results; Crystallographic data of the structure have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-241333 and CCDC-241334 — copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk].
21. Höfle, G. et al., unpublished results.
22. Straubing, R.M., Biopharmaceuticals of paclitaxel, in *Taxol®, Science Application*, Suffness, M., Ed., CRC Press, Boca Raton, FL, 1995, 238.
23. a) Chou, T.-C. et al., Desoxyepothilone B is curative against human tumor xenografts that are refractory to paclitaxel, *Proc. Natl. Acad. Sci. USA*, 95, 15798, 1998; b) Lee, C.B. et al., Total synthesis and antitumor activity of 12,13-desoxyepothilone F: An unexpected solvolysis problem at C15, mediated by remote substitution at C21, *J. Org. Chem.*, 65, 6525, 2000.
24. Tang, L. et al., Cloning and heterologous expression of the epothilone gene cluster, *Science*, 287, 640, 2000.
25. Julien, B. and Shah, S., Heterologous expression of epothilone biosynthetic genes in *Myxococcus xanthus*, *Antimicrob. Agents Chemother.*, 46, 2772, 2002.
26. Lau, J. et al., Optimizing the heterologous production of epothilone D in *Myxococcus xanthus*, *Biotechnol. Bioeng.*, 78, 280, 2002.
27. Arslanian, R.L. et al., Large-scale isolation and crystallization of epothilone D from *Myxococcus xanthus* cultures, *J. Nat. Prod.*, 65, 570, 2002.
28. Regentin, R. et al., Nutrient regulation of epothilone biosynthesis in heterologous and native production strains, *Appl. Microbiol. Biotechnol.*, 61, 451, 2003.
29. Frykman, S. et al., Modulation of epothilone analog production through media design, *J. Ind. Microbiol. Biotechnol.*, 28, 17, 2002.
30. Molnar, I. et al., The biosynthetic gene cluster for the microtubule-stabilizing agents epothilones A and B from *Sorangium cellulosum* So ce90, *Chem. Biol.*, 7, 97, 2000.
31. Julien, B. et al., Isolation and characterization of the epothilone biosynthetic gene cluster from *Sorangium cellulosum*, *Gene*, 249, 153, 2000.
32. Schupp, T. et al., World Patent 9966028-A (Dec. 23, 1999), (Novartis AG).

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use IXEMPRA™ safely and effectively. See full prescribing information for IXEMPRA™.

IXEMPRA™ *Kit* (ixabepilone) for Injection, for intravenous infusion only
Initial U.S. Approval: Year

WARNING: TOXICITY IN HEPATIC IMPAIRMENT
See full prescribing information for complete boxed warning.
IXEMPRA™ in combination with capecitabine must not be given to patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN due to increased risk of toxicity and neutropenia-related death. (4, 5.3)

INDICATIONS AND USAGE

IXEMPRA, a microtubule inhibitor, in combination with capecitabine is indicated for the treatment of metastatic or locally advanced breast cancer in patients after failure of an anthracycline and a taxane (1).
IXEMPRA as monotherapy is indicated for the treatment of metastatic or locally advanced breast cancer in patients after failure of an anthracycline, a taxane, and capecitabine (1).

DOSAGE AND ADMINISTRATION

- The recommended dose of IXEMPRA is 40 mg/m^2 infused intravenously over 3 hours every 3 weeks (2.1).
- Dose reduction is required in certain patients with elevated AST, ALT, or bilirubin (2.2, 8.6).

IXEMPRA (ixabepilone) for injection must be constituted with supplied DILUENT. The ixabepilone concentration in constituted solution is 2 mg/mL. Constituted solution must be diluted with Lactated Ringer's Injection, USP, to a final ixabepilone concentration of 0.2 mg/mL to 0.6 mg/mL. The final solution must be used within 6 hours of preparation. (2.4)

DOSAGE FORMS AND STRENGTHS

- IXEMPRA for injection, 15 mg supplied with DILUENT for IXEMPRA, 8 mL (3)
- IXEMPRA for injection, 45 mg supplied with DILUENT for IXEMPRA, 23.5 mL (3)

CONTRAINDICATIONS

- Hypersensitivity to drugs formulated with Cremophor® EL (4).
- Baseline neutrophil count <1500 cells/mm^3 or a platelet count <100,000 cells/mm^3 (4).
- Patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN must not be treated with IXEMPRA in combination with capecitabine (4).

WARNINGS AND PRECAUTIONS

- Peripheral Neuropathy: Monitor for symptoms of neuropathy, primarily sensory. Neuropathy is cumulative, generally reversible and should be managed by dose adjustment and delays (2.2, 5.1).
- Myelosuppression: Primarily neutropenia. Monitor with peripheral blood cell counts and adjust dose as appropriate (2.2, 5.2).
- Hypersensitivity reaction: Must premedicate all patients with an H_1 antagonist and an H_2 antagonist before treatment (2.3, 5.4).
- Fetal harm can occur when administered to a pregnant woman. Women should be advised not to become pregnant when taking IXEMPRA (5.5, 8.1).

ADVERSE REACTIONS

- The most common adverse reactions (≥20%) are peripheral sensory neuropathy, fatigue/asthenia, myalgia/arthralgia, alopecia, nausea, vomiting, stomatitis/mucositis, diarrhea, and musculoskeletal pain. Additional reactions occurred in ≥20% in combination treatment: palmar-plantar erythrodysesthesia syndrome, anorexia, abdominal pain, nail disorder, and constipation (6).
- Drug-associated hematologic abnormalities (>40%) include neutropenia, leukopenia, anemia, and thrombocytopenia (6).

To report SUSPECTED ADVERSE REACTIONS, contact Bristol-Myers Squibb at 1-800-721-5072 or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*

DRUG INTERACTIONS

- Inhibitors of CYP3A4 may increase plasma concentrations of ixabepilone; dose of IXEMPRA must be reduced with strong CYP3A4 inhibitors (7.1).
- Inducers of CYP3A4 may decrease plasma concentrations of ixabepilone; alternative therapeutic agents with low enzyme induction potential should be considered (7.1).

See 17 for PATIENT COUNSELING INFORMATION and FDA-Approved Patient Labeling.

Revised: Month/Year

FULL PRESCRIBING INFORMATION: CONTENTS*

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FULL PRESCRIBING INFORMATION

WARNING: TOXICITY IN HEPATIC IMPAIRMENT

IXEMPRA in combination with capecitabine is contraindicated in patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN due to increased risk of toxicity and neutropenia-related death [see *Contraindications (4)* and *Warnings and Precautions (5.3)*].

1 INDICATIONS AND USAGE

IXEMPRA is indicated in combination with capecitabine for the treatment of patients with metastatic or locally advanced breast cancer resistant to treatment with an anthracycline and a taxane, or whose cancer is taxane resistant and for whom further anthracycline therapy is contraindicated. Anthracycline resistance is defined as progression while on therapy or within 6 months in the adjuvant setting or 3 months in the metastatic setting. Taxane resistance is defined as progression while on therapy or within 12 months in the adjuvant setting or 4 months in the metastatic setting.

IXEMPRA is indicated as monotherapy for the treatment of metastatic or locally advanced breast cancer in patients whose tumors are resistant or refractory to anthracyclines, taxanes, and capecitabine.

2 DOSAGE AND ADMINISTRATION

2.1 General Dosing Information

The recommended dosage of IXEMPRA is 40 mg/m^2 administered intravenously over 3 hours every 3 weeks. Doses for patients with body surface area (BSA) greater than 2.2 m^2 should be calculated based on 2.2 m^2.

2.2 Dose Modification

Dose Adjustments During Treatment

Patients should be evaluated during treatment by periodic clinical observation and laboratory tests including complete blood cell counts. If toxicities are present, treatment should be delayed to allow recovery. Dosing adjustment guidelines for monotherapy and combination therapy are shown in Table 1. If toxicities recur, an additional 20% dose reduction should be made.

Table 1: Dose Adjustment Guidelines

IXEMPRA (Monotherapy or Combination Therapy)	**IXEMPRA Dose Modification**
Nonhematologic:	
Grade 2 neuropathy (moderate) lasting ≥7 days	Decrease the dose by 20%
Grade 3 neuropathy (severe) lasting <7 days	Decrease the dose by 20%
Grade 3 neuropathy (severe) lasting ≥7 days or disabling neuropathy	Discontinue treatment
Any grade 3 toxicity (severe) other than neuropathy	Decrease the dose by 20%
Transient grade 3 arthralgia/myalgia or fatigue	No change in dose of IXEMPRA
Grade 3 hand-foot syndrome (palmar-plantar erythrodysesthesia)	
Any grade 4 toxicity (disabling)	Discontinue treatment
Hematologic:	
Neutrophil <500 cells/mm^3 for ≥7 days	Decrease the dose by 20%
Febrile neutropenia	Decrease the dose by 20%
Platelets <25,000/mm^3 or platelets <50,000/mm^3 with bleeding	Decrease the dose by 20%
CAPECITABINE (when used in combination with IXEMPRA)	**Capecitabine Dose Modification**
Nonhematologic:	Follow Capecitabine Label
Hematologic:	
Platelets < 25,000/mm^3 or <50,000/mm^3 with bleeding	Hold for concurrent diarrhea or stomatitis until platelet count >50,000/mm^3, then continue at same dose.
Neutrophils <500 cells/mm^3 for ≥7 days or febrile neutropenia	Hold for concurrent diarrhea or stomatitis until neutrophil count >1,000 cells/mm^3, then continue at same dose.

Toxicities graded in accordance with National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE v3.0).

Re-treatment Criteria: Dose adjustments at the start of a cycle should be based on nonhematologic toxicity or blood counts from the preceding cycle following the guidelines in Table 1. Patients should not begin a new cycle of treatment unless the neutrophil count is at least 1500 cells/mm^3, the platelet count is at least 100,000 cells/mm^3, and nonhematologic toxicities have improved to grade 1 (mild) or resolved.

Dose Adjustments in Special Populations - Hepatic Impairment

Combination Therapy:

IXEMPRA in combination with capecitabine is contraindicated in patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN. Patients receiving combination treatment who have AST and ALT ≤2.5 x ULN and bilirubin ≤1 x ULN may receive the standard dose of ixabepilone (40 mg/m^2). [See *Boxed Warning, Contraindications (4), Warnings and Precautions (5.3), Use in Specific Populations (8.6).*]

Monotherapy:

Patients with hepatic impairment should be dosed with IXEMPRA based on the guidelines in Table 2. Patients with moderate hepatic impairment should be started at 20 mg/m^2, the dosage in subsequent cycles may be escalated up to, but not exceeding, 30 mg/m^2 if tolerated. Use in patients with AST or ALT >10 x ULN or bilirubin >3 is not recommended. Limited data are available for patients with baseline AST or ALT >5 x ULN. Caution should be used when treating these patients. [See *Warnings and Precautions (5.3), Use in Specific Populations (8.6).*]

Table 2: Dose Adjustments for IXEMPRA as Monotherapy in Patients with Hepatic Impairment

	Transaminase Levels		Bilirubin Levels[a]	IXEMPRA[b] (mg/m^2)
Mild	AST and ALT ≤2.5 x ULN	and	≤1 x ULN	40
	AST or ALT ≤ 10 x ULN	and	≤1 x ULN	32
Moderate	AST and ALT ≤10 x ULN	and	>1.5 x ULN - ≤3 x ULN	20 - 30

a Excluding patients whose total bilirubin is elevated due to Gilbert's disease.

b Dosage recommendations are for first course of therapy; further decreases in subsequent courses should be based on individual tolerance.

Strong CYP3A4 Inhibitors

The use of concomitant strong CYP3A4 inhibitors should be avoided (eg, ketoconazole, itraconazole, clarithromycin, atazanavir, nefazodone, saquinavir, telithromycin, ritonavir, amprenavir, indinavir, nelfinavir, delavirdine, or voriconazole). Grapefruit juice may also increase plasma concentrations of IXEMPRA and should be avoided. Based on pharmacokinetic studies, if a strong CYP3A4 inhibitor must be coadministered, a dose reduction to 20 mg/m^2 is predicted to adjust the ixabepilone AUC to the range observed without inhibitors and should be considered. If the strong inhibitor is discontinued, a washout period of approximately 1 week should be allowed before the IXEMPRA dose is adjusted upward to the indicated dose. [See *Drug Interactions (7.1).*]

2.3 Premedication

To minimize the chance of occurrence of a hypersensitivity reaction, all patients must be premedicated approximately 1 hour before the infusion of IXEMPRA with:

- An H_1 antagonist (eg, diphenhydramine 50 mg orally or equivalent) and
- An H_2 antagonist (eg, ranitidine 150 - 300 mg orally or equivalent).

Patients who experienced a hypersensitivity reaction to IXEMPRA require premedication with corticosteroids (eg, dexamethasone 20 mg intravenously, 30 minutes before infusion or orally, 60 minutes before infusion) in addition to pretreatment with H_1 and H_2 antagonists.

2.4 Instructions for Preparation and IV Administration

IXEMPRA *Kit* contains two vials, a vial labeled IXEMPRA (ixabepilone) for injection which contains ixabepilone powder and a vial containing DILUENT for IXEMPRA. Only supplied DILUENT must be used for constituting IXEMPRA (ixabepilone) for injection. IXEMPRA *Kit* must be stored in a refrigerator at 2° C - 8° C (36° F - 46° F) in the original package to protect from light. Prior to constituting IXEMPRA for injection, the *Kit* should be removed from the refrigerator and allowed to stand at room temperature for approximately 30 minutes. When the vials are first removed from the refrigerator, a white precipitate may be observed in the DILUENT vial. This precipitate will dissolve to form a clear solution once the DILUENT warms to room temperature. To allow for withdrawal losses, the vial labeled as 15 mg IXEMPRA for injection contains 16 mg of ixabepilone and the vial labeled as 45 mg IXEMPRA for injection contains 47 mg of ixabepilone. The 15-mg IXEMPRA *Kit* is supplied with a vial providing 8 mL of the DILUENT and the 45-mg IXEMPRA *Kit* is supplied with a vial providing 23.5 mL of the DILUENT. After constituting with the DILUENT, the concentration of ixabepilone is 2 mg/mL.

Please refer to Preparation and Handling Precautions [See Dosage and Administration (2.5)] before preparation.

A. To constitute:

1. With a suitable syringe, aseptically withdraw the DILUENT and slowly inject it into the IXEMPRA for injection vial. The 15-mg IXEMPRA is constituted with 8 mL of DILUENT and the 45-mg IXEMPRA is constituted with 23.5 mL of DILUENT.
2. Gently swirl and invert the vial until the powder in IXEMPRA is completely dissolved.

B. To dilute:

Before administration, the constituted solution must be further diluted only with Lactated Ringer's Injection, USP (LRI) supplied in DEHP [di-(2-ethylhexyl)phthalate] free bags. For most doses, a 250 mL bag of Lactated Ringer's Injection is sufficient. However, it is necessary to check the final infusion concentration of each dose based on the volume of Lactated Ringer's Injection to be used. The final concentration for infusion must be between 0.2 mg/mL and 0.6 mg/mL. To calculate the final infusion concentration, use the following formulas:

Total Infusion Volume = mL of Constituted Solution + mL of LRI

Final Infusion Concentration = Dose of IXEMPRA (mg)/Total Infusion Volume (mL)

1. Aseptically, withdraw the appropriate volume of constituted solution containing 2 mg of ixabepilone per mL.
2. Aseptically, transfer to an intravenous (IV) bag containing an appropriate volume of Lactated Ringer's Injection, USP to achieve the final desired concentration of ixabepilone.
3. Thoroughly mix the infusion bag by manual rotation.

The infusion solution must be administered through an appropriate in-line filter with a microporous membrane of 0.2 to 1.2 microns. DEHP-free infusion containers and administration sets must be used. Any remaining solution should be discarded according to institutional procedures for antineoplastics.

Stability

After constituting ixabepilone for injection, the constituted solution should be further diluted with Lactated Ringer's Injection as soon as possible, but may be stored in the vial (not the syringe) for a maximum of 1 hour at room temperature and room light. Once diluted with Lactated Ringer's Injection, USP the solution is stable at room temperature and room light for a maximum of 6 hours. Administration of diluted

IXEMPRA must be completed within this 6-hour period. Lactated Ringer's Injection USP is specified because it has a pH range of 6 to 7.5, which is required to maintain IXEMPRA stability. Other diluents should not be used with IXEMPRA.

2.5 Preparation and Handling Precautions

Procedures for proper handling and disposal of antineoplastic drugs [see *References (15)*] should be followed. To minimize the risk of dermal exposure, impervious gloves should be worn when handling vials containing IXEMPRA, regardless of the setting, including unpacking and inspection, transport within a facility, and dose preparation and administration.

3 DOSAGE FORMS AND STRENGTHS

IXEMPRA, for injection 15 mg supplied with DILUENT for IXEMPRA, 8 mL.

IXEMPRA, for injection 45 mg supplied with DILUENT for IXEMPRA, 23.5 mL.

4 CONTRAINDICATIONS

IXEMPRA is contraindicated in patients with a history of a severe (CTC grade 3/4) hypersensitivity reaction to agents containing Cremophor® EL or its derivatives (eg, polyoxyethylated castor oil) [see *Warnings and Precautions (5.4)*].

IXEMPRA is contraindicated in patients who have a neutrophil count <1500 cells/mm^3 or a platelet count <100,000 cells/mm^3 [see *Warnings and Precautions (5.2)*].

IXEMPRA in combination with capecitabine is contraindicated in patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN [see *Boxed Warning* and *Warnings and Precautions (5.3)*].

5 WARNINGS AND PRECAUTIONS

5.1 Peripheral Neuropathy

Peripheral neuropathy was common (see Table 3). Patients treated with IXEMPRA should be monitored for symptoms of neuropathy, such as burning sensation, hyperesthesia, hypoesthesia, paresthesia, discomfort, or neuropathic pain. Neuropathy occurred early during treatment; ~75% of new onset or worsening neuropathy occurred during the first 3 cycles. Patients experiencing new or worsening symptoms may require a reduction or delay in the dose of IXEMPRA [see *Dosage and Administration (2.2)*]. In clinical studies, peripheral neuropathy was managed through dose reductions, dose delays and treatment discontinuation. Neuropathy was the most frequent cause of treatment

discontinuation due to drug toxicity. In Studies 046 and 081, 80% and 87%, respectively, of patients with peripheral neuropathy who received IXEMPRA had improvement or no worsening of their neuropathy following dose reduction. For patients with grade 3/4 neuropathy in Studies 046 and 081, 76% and 79%, respectively, had documented improvement to baseline or grade 1, twelve weeks after onset.-

Table 3: Treatment-related Peripheral Neuropathy

	IXEMPRA with capecitabine Study 046	IXEMPRA as monotherapy Study 081
Peripheral neuropathy (all grades)[a,b]	67%	63%
Peripheral neuropathy (grades 3/4)[a,b]	23%	14%
Discontinuation due to neuropathy	21%	6%
Median number of cycles to onset of grade 3/4 neuropathy	4	4
Median time to improvement of grade 3/4 neuropathy to baseline or to grade 1	6.0 weeks	4.6 weeks

[a] Sensory and motor neuropathy combined.

[b] 24% and 27 % of patients in 046 and 081, respectively had preexisting neuropathy (grade 1).

A pooled analysis of 945 cancer patients treated with IXEMPRA indicated that patients with diabetes mellitus may be at increased risk of severe neuropathy. The presence of grade 1 neuropathy and prior therapy with neurotoxic chemotherapy agents did not predict either the development or worsening of neuropathy. Patients with moderate to severe neuropathy (grade 2 or greater) were excluded from studies with IXEMPRA. Caution should be used when treating patients with diabetes mellitus or existing moderate to severe neuropathy.

5.2 Myelosuppression

Myelosuppression is dose-dependent and primarily manifested as neutropenia. In clinical studies, grade 4 neutropenia (<500 cells/mm^3) occurred in 36% of patients treated with IXEMPRA in combination with capecitabine and 23% of patients treated with IXEMPRA monotherapy. Febrile neutropenia and infection with neutropenia were reported in 5% and 6% of patients treated with IXEMPRA in combination with capecitabine, respectively, and 3% and 5% of patients treated with IXEMPRA as monotherapy, respectively. Neutropenia-related death occurred in 1.9% of 414 patients with normal hepatic function or mild hepatic impairment treated with IXEMPRA in

combination with capecitabine. The rate of neutropenia-related deaths was higher (29%, 5 out of 17) in patients with AST or ALT >2.5 x ULN or bilirubin >1.5 x ULN. [See *Boxed Warning, Contraindications (4),* and *Warnings and Precautions (5.3).*] Neutropenia-related death occurred in 0.4% of 240 patients treated with IXEMPRA as monotherapy. No neutropenia-related deaths were reported in 24 patients with AST or ALT >2.5 x ULN or bilirubin >1.5 x ULN treated with IXEMPRA monotherapy. IXEMPRA must not be administered to patients with a neutrophil count <1500 cells/mm^3. To monitor for myelosuppression, frequent peripheral blood cell counts are recommended for all patients receiving IXEMPRA. Patients who experience severe neutropenia or thrombocytopenia should have their dose reduced [see *Dosage and Administration (2.2)*].

5.3 Hepatic Impairment

Patients with baseline AST or ALT >2.5 x ULN or bilirubin >1.5 x ULN experienced greater toxicity than patients with baseline AST or ALT ≤2.5 x ULN or bilirubin ≤1.5 x ULN when treated with IXEMPRA at 40 mg/m^2 in combination with capecitabine or as monotherapy in breast cancer studies. In combination with capecitabine, the overall frequency of grade 3/4 adverse reactions, febrile neutropenia, serious adverse reactions, and toxicity related deaths was greater [see *Warnings and Precautions (5.2)*]. With monotherapy, grade 4 neutropenia, febrile neutropenia, and serious adverse reactions were more frequent. The safety and pharmacokinetics of IXEMPRA as monotherapy were evaluated in a dose escalation study in 56 patients with varying degrees of hepatic impairment. Exposure was increased in patients with elevated AST or bilirubin [see *Use in Specific Populations (8.6)*].

IXEMPRA in combination with capecitabine is contraindicated in patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN due to increased risk of toxicity and neutropenia-related death [see *Boxed Warning, Contraindications (4),* and *Warnings and Precautions (5.2)*]. Patients who are treated with IXEMPRA as monotherapy should receive a reduced dose depending on the degree of hepatic impairment [see *Dosage and Administration (2.2)*]. Use in patients with AST or ALT >10 x ULN or bilirubin >3 x ULN is not recommended. Limited data are available for patients with AST or ALT >5 x ULN. Caution should be used when treating these patients [see *Dosage and Administration (2.2)*].

5.4 Hypersensitivity Reactions

Patients with a history of a severe hypersensitivity reaction to agents containing Cremophor® EL or its derivatives (eg, polyoxyethylated castor oil) should not be treated

with IXEMPRA. All patients should be premedicated with an H_1 and an H_2 antagonist approximately 1 hour before IXEMPRA infusion and be observed for hypersensitivity reactions (e.g., flushing, rash, dyspnea and bronchospasm). In case of severe hypersensitivity reactions, infusion of IXEMPRA should be stopped and aggressive supportive treatment (eg, epinephrine, corticosteroids) started. Of the 1323 patients treated with IXEMPRA in clinical studies, 9 patients (1%) had experienced severe hypersensitivity reactions (including anaphylaxis). Three of the 9 patients were able to be retreated. Patients who experience a hypersensitivity reaction in one cycle of IXEMPRA must be premedicated in subsequent cycles with a corticosteroid in addition to the H_1 and H_2 antagonists, and extension of the infusion time should be considered [see *Dosage and Administration (2.3)* and *Contraindications (4)*].

5.5 Pregnancy

Pregnancy Category D.

IXEMPRA may cause fetal harm when administered to pregnant women. There are no adequate and well-controlled studies with IXEMPRA in pregnant women. Women should be advised not to become pregnant when taking IXEMPRA. If this drug is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Ixabepilone was studied for effects on embryo-fetal development in pregnant rats and rabbits given IV doses of 0.02, 0.08, and 0.3 mg/kg/day and 0.01, 0.03, 0.11 and 0.3 mg/kg/day, respectively. There were no teratogenic effects. In rats, an increase in resorptions and post-implantation loss and a decrease in the number of live fetuses and fetal weight was observed at the maternally toxic dose of 0.3 mg/kg/day (approximately one-tenth the human clinical exposure based on AUC). Abnormalities included a reduced ossification of caudal vertebrae, sternebrae, and metacarpals. In rabbits, ixabepilone caused maternal toxicity (death) and embryo-fetal toxicity (resorptions) at 0.3 mg/kg/day (approximately one-tenth the human clinical dose based on body surface area). No fetuses were available at this dose for evaluation.

5.6 Cardiac Adverse Reactions

The frequency of cardiac adverse reactions (myocardial ischemia and ventricular dysfunction) was higher in the IXEMPRA in combination with capecitabine (1.9%) than in the capecitabine alone (0.3%) treatment group. Supraventricular arrhythmias were observed in the combination arm (0.5%) and not in the capecitabine alone arm. Caution should be exercised in patients with a history of cardiac disease. Discontinuation of

IXEMPRA should be considered in patients who develop cardiac ischemia or impaired cardiac function.

5.7 Potential for Cognitive Impairment from Excipients

Since IXEMPRA contains dehydrated alcohol USP, consideration should be given to the possibility of central nervous system and other effects of alcohol [see *Description (11)*].

6 ADVERSE REACTIONS

The following adverse reactions are discussed in greater detail in other sections.

- Peripheral neuropathy [see *Warnings and Precautions (5.1)*]
- Myelosuppression [see *Warnings and Precautions (5.2)*]
- Hypersensitivity reactions [see *Warnings and Precautions (5.4)*]

Because clinical trials are conducted under widely varying conditions, the adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in other clinical trials and may not reflect the rates observed in clinical practice.

Unless otherwise specified, assessment of adverse reactions is based on one randomized study (Study 046) and one single-arm study (Study 081). In Study 046, 369 patients with metastatic breast cancer were treated with IXEMPRA 40 mg/m^2 administered intravenously over 3 hours every 21 days, combined with capecitabine 1000 mg/m^2 twice daily for 2 weeks followed by a 1-week rest period. Patients treated with capecitabine as monotherapy (n=368) in this study received 1250 mg/m^2 twice daily for 2 weeks every 21 days. In Study 081, 126 patients with metastatic or locally advanced breast cancer were treated with IXEMPRA 40 mg/m^2 administered intravenously over 3 hours every 3 weeks.

The most common adverse reactions (≥20%) reported by patients receiving IXEMPRA were peripheral sensory neuropathy, fatigue/asthenia, myalgia/arthralgia, alopecia, nausea, vomiting, stomatitis/mucositis, diarrhea, and musculoskeletal pain. The following additional reactions occurred in ≥20% in combination treatment: palmar-plantar erythrodysesthesia (hand-foot) syndrome, anorexia, abdominal pain, nail disorder, and constipation. The most common hematologic abnormalities (>40%) include neutropenia, leukopenia, anemia, and thrombocytopenia.

Table 4 presents nonhematologic adverse reactions reported in 5% or more of patients. Hematologic abnormalities are presented separately in Table 5.

Table 4: Nonhematologic Drug-related Adverse Reactions Occurring in at Least 5% of Patients with Metastatic or Locally Advanced Breast Cancer Treated with IXEMPRA

	Study 046				Study 081	
	IXEMPRA with capecitabine n=369		Capecitabine n=368		IXEMPRA monotherapy n=126	
System Organ Class/ Preferred Term	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)
Infections and Infestations						
Upper respiratory tract infection	4	0	3	0	6	0
Blood and Lymphatic System Disorders						
Febrile neutropenia	5	4	1	1	3	3[d]
Immune System Disorders						
Hypersensitivity	2	1[d]	0	0	5	1[d]
Metabolism and Nutrition Disorders						
Anorexia	34	3[d]	15	1[d]	19	2[d]
Dehydration	5	2	2	<1[d]	2	1[d]
Psychiatric						
Insomnia	9	<1[d]	2	0	5	0
Nervous System Disorders						
Peripheral neuropathy						
Sensory neuropathy[b,e]	65	21	16	0	62	14
Motor neuropathy	16	5[d]	<1	0	10	1[d]
Headache	8	<1[d]	3	0	11	0
Taste disorder	12	0	4	0	6	0
Dizziness	8	1[d]	5	1[d]	7	0
Eye Disorders						
Lacrimation increased	5	0	4	<1[d]	4	0
Vascular Disorders						
Hot flush	5	0	2	0	6	0
Respiratory, Thoracic, and Mediastinal Disorders						

Table 4: Nonhematologic Drug-related Adverse Reactions Occurring in at Least 5% of Patients with Metastatic or Locally Advanced Breast Cancer Treated with IXEMPRA

	Study 046				Study 081	
	IXEMPRA with capecitabine n=369		Capecitabine n=368		IXEMPRA monotherapy n=126	
System Organ Class/ Preferred Term	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)
Dyspnea	7	1	4	1	9	1[d]
Cough	6	0	2	0	2	0
Gastrointestinal Disorders						
Nausea	53	3[d]	40	2[d]	42	2[d]
Vomiting	39	4[d]	24	2	29	1[d]
Stomatitis/mucositis	31	4	20	3[d]	29	6
Diarrhea	44	6[d]	39	9	22	1[d]
Constipation	22	0	6	<1[d]	16	2[d]
Abdominal pain	24	2[d]	14	1[d]	13	2[d]
Gastroesophageal reflux disease	7	1[d]	8	0	6	0
Skin and Subcutaneous Tissue Disorders						
Alopecia	31	0	3	0	48	0
Skin rash	17	1[d]	7	0	9	2[d]
Nail disorder	24	2[d]	10	<1[d]	9	0
Palmar-plantar erythrodysesthesia syndrome[b,f]	64	18[d]	63	17[d]	8	2[d]
Pruritus	5	0	2	0	6	1[d]
Skin exfoliation	5	<1[d]	3	0	2	0
Skin hyperpigmentation	11	0	14	0	2	0
Musculoskeletal, Connective Tissue, and Bone Disorders						
Myalgia/arthralgia	39	8[d]	5	<1[d]	49	8[d]
Musculoskeletal pain	23	2[d]	5	0	20	3[d]

Table 4: Nonhematologic Drug-related Adverse Reactions Occurring in at Least 5% of Patients with Metastatic or Locally Advanced Breast Cancer Treated with IXEMPRA

	Study 046				Study 081	
	IXEMPRA with capecitabine n=369		Capecitabine n=368		IXEMPRA monotherapy n=126	
System Organ Class/ Preferred Term	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)	Total (%)	Grade 3/4 (%)
General Disorders and Administrative Site Conditions						
Fatigue/asthenia	60	16	29	4	56	13
Edema	8	0	5	<1[d]	9	1[d]
Pyrexia	10	1[d]	4	0	8	1[d]
Pain	9	1[d]	2	0	8	3[d]
Chest pain	4	1[d]	<1	0	5	1[d]
Investigations						
Weight decreased	11	0	3	0	6	0

System organ class presented as outlined in Guidelines for Preparing Core Clinical Safety Information on Drugs by the Council for International Organizations of Medical Sciences (CIOMS).

A composite of multiple MedDRA Preferred Terms.

NCI CTC grading for febrile neutropenia ranges from Grade 3 to 5. Three patients (1%) experienced Grade 5 (fatal) febrile neutropenia. Other neutropenia-related deaths (9) occurred in the absence of reported febrile neutropenia [see *Warnings and Precautions* (5.2)].

d No grade 4 reports.

e Peripheral sensory neuropathy (graded with the NCI CTC scale) was defined as the occurrence of any of the following: areflexia, burning sensation, dysesthesia, hyperesthesia, hypoesthesia, hyporeflexia, neuralgia, neuritis, neuropathy, neuropathy peripheral, neurotoxicity, painful response to normal stimuli, paresthesia, pallanesthesia, peripheral sensory neuropathy, polyneuropathy, polyneuropathy toxic and sensorimotor disorder.

Peripheral motor neuropathy was defined as the occurrence of any of the following: multifocal motor neuropathy, neuromuscular toxicity, peripheral motor neuropathy, and peripheral sensorimotor neuropathy.

f Palmar-plantar erythrodysesthesia (hand-foot syndrome) was graded on a 1-3 severity scale in Study 046.

Table 5: Hematologic Abnormalities in Patients with Metastatic or Locally Advanced Breast Cancer Treated with IXEMPRA

	Study 046		Study 081
	IXEMPRA with capecitabine n=369	Capecitabine n=368	IXEMPRA monotherapy n=126

Hematology Parameter	Grade 3 (%)	Grade 4 (%)	Grade 3 (%)	Grade 4 (%)	Grade 3 (%)	Grade 4 (%)
Neutropenia	32	36	9	2	31	23
Leukopenia (WBC)	41	16	5	1	36	13
Anemia (Hgb)	8	2	4	1	6	2
Thrombocytopenia	5	3	2	2	5	2

[a] G-CSF (granulocyte colony stimulating factor) or GM-CSF (granulocyte macrophage stimulating factor) was used in 20% and 17% of patients who received IXEMPRA in Study 046 and Study 081, respectively.

The following serious adverse reactions were also reported in 1323 patients treated with IXEMPRA as monotherapy or in combination with other therapies in Phase 2 and 3 studies.

Infections and Infestations: sepsis, pneumonia, infection, neutropenic infection, urinary tract infection, bacterial infection, enterocolitis, laryngitis, lower respiratory tract infection

Blood and Lymphatic System Disorders: coagulopathy, lymphopenia

Metabolism and Nutrition Disorders: hyponatremia, metabolic acidosis, hypokalemia, hypovolemia

Nervous System Disorders: cognitive disorder, syncope, cerebral hemorrhage, abnormal coordination, lethargy

Cardiac Disorders: myocardial infarction, supraventricular arrhythmia, left ventricular dysfunction, angina pectoris, atrial flutter, cardiomyopathy, myocardial ischemia

Vascular Disorders: hypotension, thrombosis, embolism, hemorrhage, hypovolemic shock, vasculitis

Respiratory, Thoracic, and Mediastinal Disorders: pneumonitis, hypoxia, respiratory failure, acute pulmonary edema, dysphonia, pharyngolaryngeal pain

Gastrointestinal Disorders: ileus, colitis, impaired gastric emptying, esophagitis, dysphagia, gastritis, gastrointestinal hemorrhage

Hepatobiliary Disorders: acute hepatic failure, jaundice

Skin and Subcutaneous Tissue Disorders: erythema multiforme

Musculoskeletal, Connective Tissue Disorders, and Bone Disorders: muscular weakness, muscle spasms, trismus

Renal and Urinary Disorders: nephrolithiasis, renal failure

General Disorders and Administration Site Conditions: chills

Investigations: increased transaminases, increased blood alkaline phosphatase, increased gamma-glutamyltransferase.

7 DRUG INTERACTIONS

7.1 Effect of Other Drugs on Ixabepilone

Drugs That May Increase Ixabepilone Plasma Concentrations

CYP3A4 Inhibitors: Co-administration of ixabepilone with ketoconazole, a potent CYP3A4 inhibitor, increased ixabepilone AUC by 79% compared to ixabepilone treatment alone. If alternative treatment cannot be administered, a dose adjustment should be considered. The effect of mild or moderate inhibitors (eg, erythromycin, fluconazole, or verapamil) on exposure to ixabepilone has not been studied. Therefore, caution should be used when administering mild or moderate CYP3A4 inhibitors during treatment with IXEMPRA, and alternative therapeutic agents that do not inhibit CYP3A4 should be considered. Patients receiving CYP3A4 inhibitors during treatment with IXEMPRA should be monitored closely for acute toxicities (eg, frequent monitoring of peripheral blood counts between cycles of IXEMPRA). [*See Dosage and Administration (2.2).*]

Drugs That May Decrease Ixabepilone Plasma Concentrations

CYP3A4 Inducers: IXEMPRA is a CYP3A4 substrate. Strong CYP3A4 inducers (eg, dexamethasone, phenytoin, carbamazepine, rifampin, rifampicin, rifabutin, and phenobarbital) may decrease ixabepilone concentrations leading to subtherapeutic levels. Therefore, therapeutic agents with low enzyme induction potential should be considered for coadministration with IXEMPRA. St. John's Wort may decrease ixabepilone plasma concentrations unpredictably and should be avoided.

7.2 Effect of Ixabepilone on Other Drugs

Ixabepilone does not inhibit CYP enzymes at relevant clinical concentrations and is not expected to alter the plasma concentrations of other drugs [see *Clinical Pharmacology (12.3)*].

7.3 Capecitabine

In patients with cancer who received ixabepilone (40 mg/m^2) in combination with capecitabine (1000 mg/m^2), ixabepilone Cmax decreased by 19%, capecitabine Cmax decreased by 27%, and 5-fluorouracil AUC increased by 14%, as compared to ixabepilone or capecitabine administered separately. The interaction is not clinically significant given that the combination treatment is supported by efficacy data.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D [See *Warnings and Precautions (5.5)*]

8.3 Nursing Mothers

It is not known whether ixabepilone is excreted into human milk. Following intravenous administration of radiolabeled ixabepilone to rats on days 7 to 9 postpartum, concentrations of radioactivity in milk were comparable with those in plasma and declined in parallel with the plasma concentrations. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from ixabepilone, a decision must be made whether to discontinue nursing or to discontinue IXEMPRA taking into account the importance of the drug to the mother.

8.4 Pediatric Use

The safety and effectiveness of IXEMPRA in pediatric patients have not been established.

8.5 Geriatric Use

Clinical studies of IXEMPRA did not include sufficient numbers of subjects aged sixty five and over to determine whether they respond differently from younger subjects.

Forty-five of 431 patients treated with IXEMPRA in combination with capecitabine were ≥65 years of age and 3 patients were ≥75. Overall, the incidence of grade 3/4 adverse reactions were higher in patients ≥65 years of age versus those <65 years of age (82% versus 68%) including grade 3/4 stomatitis (9% versus 1%), diarrhea (9% versus 6%), palmar-plantar erythrodysesthesia syndrome (27% versus 20%), peripheral neuropathy (24% versus 22%), febrile neutropenia (9% versus 3%), fatigue (16% versus 12%), and asthenia (11% versus 6%). Toxicity-related deaths occurred in 2 (4.7%) of 43 patients ≥65 years with normal baseline hepatic function or mild impairment.

Thirty-two of 240 breast cancer patients treated with IXEMPRA as monotherapy were ≥65 years of age and 6 patients were ≥75. No overall differences in safety were observed in these patients compared to those <65 years of age.

8.6 Hepatic Impairment

IXEMPRA was evaluated in 56 patients with mild to severe hepatic impairment defined by bilirubin levels and AST levels. Compared to patients with normal hepatic function (n=17), the area under the curve ($AUC_{0\text{-infinity}}$) of ixabepilone increased by:

- 22% in patients with a) bilirubin >1 – 1.5 x ULN **or** b) AST >ULN but bilirubin <1.5 x ULN;
- 30% in patients with bilirubin >1.5 – 3 x ULN and any AST level; and
- 81% in patients with bilirubin >3 x ULN and any AST level.

Doses of 10 and 20 mg/m^2 as monotherapy were tolerated in 17 patients with severe hepatic impairment (bilirubin >3 x ULN).

IXEMPRA in combination with capecitabine must not be given to patients with AST or ALT >2.5 x ULN or bilirubin >1 x ULN [see *Boxed Warning, Contraindications (4),* and *Warnings and Precautions (5.3)*]. Dose reduction is recommended when administering IXEMPRA as monotherapy to patients with hepatic impairment [see *Dosage and Administration (2.3)*]. Because there is a need for dosage adjustment based upon hepatic function, assessment of hepatic function is recommended before initiation of IXEMPRA and periodically thereafter.

8.7 Renal Impairment

Ixabepilone is minimally excreted via the kidney. No controlled pharmacokinetic studies were conducted with IXEMPRA in patients with renal impairment. IXEMPRA in combination with capecitabine has not been evaluated in patients with calculated creatinine clearance of <50 mL/min. IXEMPRA as monotherapy has not been evaluated in patients with creatinine >1.5 times ULN. In a population pharmacokinetic analysis of IXEMPRA as monotherapy, there was no meaningful effect of mild and moderate renal insufficiency (CrCL >30 mL/min) on the pharmacokinetics of ixabepilone.

10 OVERDOSAGE

One case of overdose of IXEMPRA has been reported. The patient mistakenly received 100 mg/m^2 (total dose 185 mg) and was admitted to the hospital for observation. The patient experienced myalgia (grade 1) and fatigue (grade 1) one day after infusion and was treated with a centrally acting analgesic. The patient recovered and was discharged without incident.

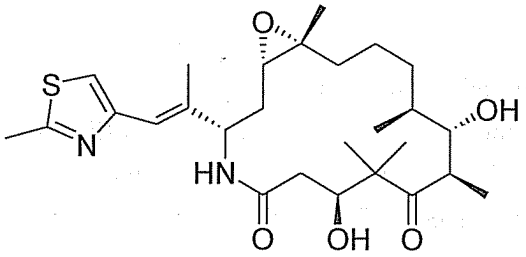
There is no known antidote for overdosage of IXEMPRA. In case of overdosage, the patient should be closely monitored, and supportive treatment should be administered.

Management of overdose should include supportive medical interventions to treat the presenting clinical manifestations.

11 DESCRIPTION

IXEMPRA (ixabepilone) is a microtubule inhibitor belonging to a class of antineoplastic agents, the epothilones and their analogs. The epothilones are isolated from the myxobacterium *Sorangium cellulosum.* Ixabepilone is a semisynthetic analog of epothilone B, a 16-membered polyketide macrolide, with a chemically modified lactam substitution for the naturally existing lactone.

The chemical name for ixabepilone is (1*S*,3*S*,7*S*,10*R*,11*S*,12*S*,16*R*)-7,11-dihydroxy-8,8,10,12,16-pentamethyl-3-[(1*E*)-1-methyl-2-(2-methyl-4-thiazolyl)ethenyl]-17-oxa-4-azabicyclo[14.1.0] heptadecane-5,9-dione, and it has a molecular weight of 506.7. Ixabepilone has the following structural formula:



IXEMPRA (ixabepilone) for Injection is intended for intravenous infusion only after constitution with the supplied DILUENT and after further dilution with Lactated Ringer's Injection, USP. IXEMPRA (ixabepilone) for injection is supplied as a sterile, non-pyrogenic single-use vial containing 15 mg or 45 mg ixabepilone as lyophilized white powder. The DILUENT for IXEMPRA is a sterile, non-pyrogenic solution of 52.8% (w/v) purified polyoxyethylated castor oil and 39.8% (w/v) dehydrated alcohol, USP. The IXEMPRA (ixabepilone) for injection and the DILUENT for IXEMPRA are co-packaged and supplied as IXEMPRA *Kit.*

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Ixabepilone is a semi-synthetic analog of epothilone B. Ixabepilone binds directly to β-tubulin subunits on microtubules, leading to suppression of microtubule dynamics. Ixabepilone suppresses the dynamic instability of αβ-II and αβ-III microtubules. Ixabepilone possesses low *in vitro* susceptibility to multiple tumor resistance mechanisms

including efflux transporters, such as MRP-1 and P-glycoprotein (P-gp). Ixabepilone blocks cells in the mitotic phase of the cell division cycle, leading to cell death.

12.2 Pharmacodynamics

In cancer patients, ixabepilone has a plasma concentration-dependent effect on tubulin dynamics in peripheral blood mononuclear cells that is observed as the formation of microtubule bundles. Ixabepilone has antitumor activity *in vivo* against multiple human tumor xenografts, including drug-resistant types that overexpress P-gp, MRP-1, and βIII tubulin isoforms, or harbor tubulin mutations. Ixabepilone is active in xenografts that are resistant to multiple agents including taxanes, anthracyclines, and vinca alkaloids. Ixabepilone demonstrated synergistic antitumor activity in combination with capecitabine *in vivo*. In addition to direct antitumor activity, ixabepilone has antiangiogenic activity.

12.3 Pharmacokinetics

Absorption

Following administration of a single 40 mg/m^2 dose of TRADNAME in patients with cancer, the mean Cmax was 252 ng/mL (coefficient of variation, CV = 56%) and the mean AUC was 2143 ng hr/mL (CV 48%). Typically Cmax occurred at the end of the 3 hour infusion. In cancer patients, the pharmacokinetics of ixabepilone were linear at doses of 15 to 57 mg/m^2.

Distribution

The mean volume of distribution of 40 mg/m^2 ixabepilone at steady-state was in excess of 1000 L. *In vitro*, the binding of ixabepilone to human serum proteins ranged from 67 to 77%, and the blood-to-plasma concentration ratios in human blood ranged from 0.65 to 0.85 over a concentration range of 50 to 5000 ng/mL.

Metabolism

Ixabepilone is extensively metabolized in the liver. *In vitro* studies indicated that the main route of oxidative metabolism of ixabepilone is via CYP3A4. More than 30 metabolites of ixabepilone are excreted into human urine and feces. No single metabolite accounted for more than 6% of the administered dose. The biotransformation products generated from ixabepilone by human liver microsomes were not active when tested for *in vitro* cytotoxicity against a human tumor cell line.

In vitro studies using human liver microsomes indicate that clinically relevant concentrations of ixabepilone do not inhibit CYP3A4, CYP1A2, CYP2A6, CYP2B6,

CYP2C8, CYP2C9, CYP2C19, or CYP2D6. Ixabepilone does not induce the activity or the corresponding mRNA levels of CYP1A2, CYP2B6, CYP2C9, or CYP3A4 in cultured human hepatocytes at clinically relevant concentrations. Therefore, it is unlikely that ixabepilone will affect the plasma levels of drugs that are substrates of CYP enzymes.

Elimination

Ixabepilone is eliminated primarily as metabolized drug. After an intravenous ^{14}[C]-ixabepilone dose to patients, approximately 86% of the dose was eliminated within 7 days in feces (65% of the dose) and in urine (21% of the dose). Unchanged ixabepilone accounted for approximately 1.6% and 5.6% of the dose in feces and urine, respectively. Ixabepilone has a terminal elimination half-life of approximately 52 hours. No accumulation in plasma is expected for ixabepilone administered every 3 weeks.

Effects of Age, Gender, and Race

Based upon a population pharmacokinetic analysis in 676 cancer patients, gender, race, and age do not have meaningful effects on the pharmacokinetics of ixabepilone.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity studies with ixabepilone have not been conducted. Ixabepilone did not induce mutations in the microbial mutagenesis (Ames) assay and was not clastogenic in an *in vitro* cytogenetic assay using primary human lymphocytes. Ixabepilone was clastogenic (induction of micronuclei) in the *in vivo* rat micronucleus assay at doses $\geq$0.625 mg/kg/day.

There were no effects on male or female rat mating or fertility at doses up to 0.2 mg/kg/day in both males and females (approximately one-fifteenth the expected human clinical exposure based on AUC). The effect of ixabepilone on human fertility is unknown. However, when rats were given an IV infusion of ixabepilone during breeding and through the first 7 days of gestation, a significant increase in resorptions and pre- and post-implantation loss and a decrease in the number of corpora lutea was observed at 0.2 mg/kg/day. Testicular atrophy or degeneration was observed in 6-month rat and 9-month dog studies when ixabepilone was given every 21 days at intravenous doses of 6.7 mg/kg (40 mg/m^2) in rats (approximately 2.1 times the expected clinical exposure based on AUC) and 0.5 and 0.75 mg/kg (10 and 15 mg/m^2) in dogs (approximately 0.2 and 0.4 times the expected clinical exposure based on AUC).

13.2 Animal Toxicology

Overdose

In rats, single intravenous doses of ixabepilone from 60 to 180 mg/m^2 (mean AUC values ≥8156 ng•h/mL) were associated with mortality occurring between 5 and 14 days after dosing, and toxicity was principally manifested in the gastrointestinal, hematopoietic (bone-marrow), lymphatic, peripheral-nervous, and male-reproductive systems. In dogs, a single intravenous dose of 100 mg/m^2 (mean AUC value of 6925 ng•h/mL) was markedly toxic, inducing severe gastrointestinal toxicity and death 3 days after dosing.

14 CLINICAL STUDIES

Combination Therapy

In an open-label, multicenter, multinational, randomized trial of 752 patients with metastatic or locally advanced breast cancer, the efficacy and safety of IXEMPRA (40 mg/m^2 every 3 weeks) in combination with capecitabine (at 1000 mg/m^2 twice daily for 2 weeks followed by 1 week rest) were assessed in comparison with capecitabine as monotherapy (at 1250 mg/m^2 twice daily for 2 weeks followed by 1 week rest). Patients were previously treated with anthracyclines and taxanes. Patients were required to have demonstrated tumor progression or resistance to taxanes and anthracyclines as follows:

- tumor progression within 3 months of the last anthracycline dose in the metastatic setting or recurrence within 6 months in the adjuvant or neoadjuvant setting, and
- tumor progression within 4 months of the last taxane dose in the metastatic setting or recurrence within 12 months in the adjuvant or neoadjuvant setting.

For anthracyclines, patients who received a minimum cumulative dose of 240 mg/m^2 of doxorubicin or 360 mg/m^2 of epirubicin were also eligible.

Sixty-seven percent of patients were White, 23% were Asian and 3% were Black. Both arms were evenly matched with regards to race, age (median 53 years), baseline performance status (Karnofsky 70-100%), and receipt of prior adjuvant or neo-adjuvant chemotherapy (75%). Tumors were ER-positive in 47% of patients, ER-negative in 43%, HER2-positive in 15%, HER2-negative in 61%, and ER-negative, PR-negative, HER2-negative in 25%. The baseline disease characteristics and previous therapies for all patients (n=752) are shown in Table 6.

Table 6: Baseline Disease Characteristics and Previous Therapies

	IXEMPRA with capecitabine n=375	Capecitabine n=377
Site of disease		
Visceral disease (liver or lung)	316 (84%)	315 (84%)
Liver	245 (65%)	228 (61%)
Lung	180 (48%)	174 (46%)
Lymph node	250 (67%)	249 (66%)
Bone	168 (45%)	162 (43%)
Skin/soft tissue	60 (16%)	62 (16%)
Number of prior chemotherapy regimens in metastatic setting		
0	27 (7%)	33 (9%)
1	179 (48%)	184 (49%)
2	152 (41%)	138 (37%)
≥3	17 (5%)	22 (6%)
Anthracycline resistance	164 (44%)	165 (44%)
Taxane Resistance		
Neoadjuvant/adjuvant setting	40 (11%)	44 (12%)
Metastatic setting	327 (87%)	319 (85%)

For IXEMPRA plus capecitabine versus capecitabine only, prior treatment in the metastatic setting included cyclophosphamide (25% vs. 23%), fluorouracil (22% vs. 16%), vinorelbine (11% vs. 12%), gemcitabine (9% each arm), carboplatin (9% vs. 7%), liposomal doxorubicin (3% each arm), and cisplatin (2% vs. 3%).

Tumor progression within 3 months in the metastatic setting or recurrence within 6 months in the adjuvant or neoadjuvant setting.

24% and 21% of patients had received 2 or more taxane-containing regimens in the combination and single agent treatment groups, respectively.

The patients in the combination treatment group received a median of 5 cycles of treatment and patients in the capecitabine monotherapy treatment group received a median of 4 cycles of treatment.

The primary endpoint of the study was progression-free survival (PFS) defined as time from randomization to radiologic progression as determined by Independent Radiologic Review (IRR), clinical progression of measurable skin lesions or death from any cause. Other study endpoints included objective tumor response based on Response Evaluation Criteria in Solid Tumors (RECIST), time to response, response duration, and overall survival. The data for overall survival analysis are not mature.

IXEMPRA in combination with capecitabine resulted in a statistically significant improvement in PFS compared to capecitabine. The results of the study are presented in Table 7 and Figure 1.

Table 7: Efficacy of IXEMPRA in Combination with Capecitabine vs Capecitabine Alone – Intent-to-Treat Analysis

Efficacy Parameter	IXEMPRA with Capecitabine n=375	Capecitabine n=377
PFS		
Number of events	242	256
Median	5.7 months	4.1 months
(95% CI)	(4.8 - 6.7)	(3.1 - 4.3)
Hazard Ratio (95% CI)	0.69 (0.58 - 0.83)	
p-value[c] (Log rank)	<0.0001	
Objective Tumor Response Rate (95% CI)	34.7% (29.9 - 39.7)	14.3% (10.9 - 18.3)
p-value[c] (CMH)[d]	<0.0001	
Duration of Response, Median (95% CI)	6.4 months (5.6 - 7.1)	5.6 months (4.2 - 7.5)

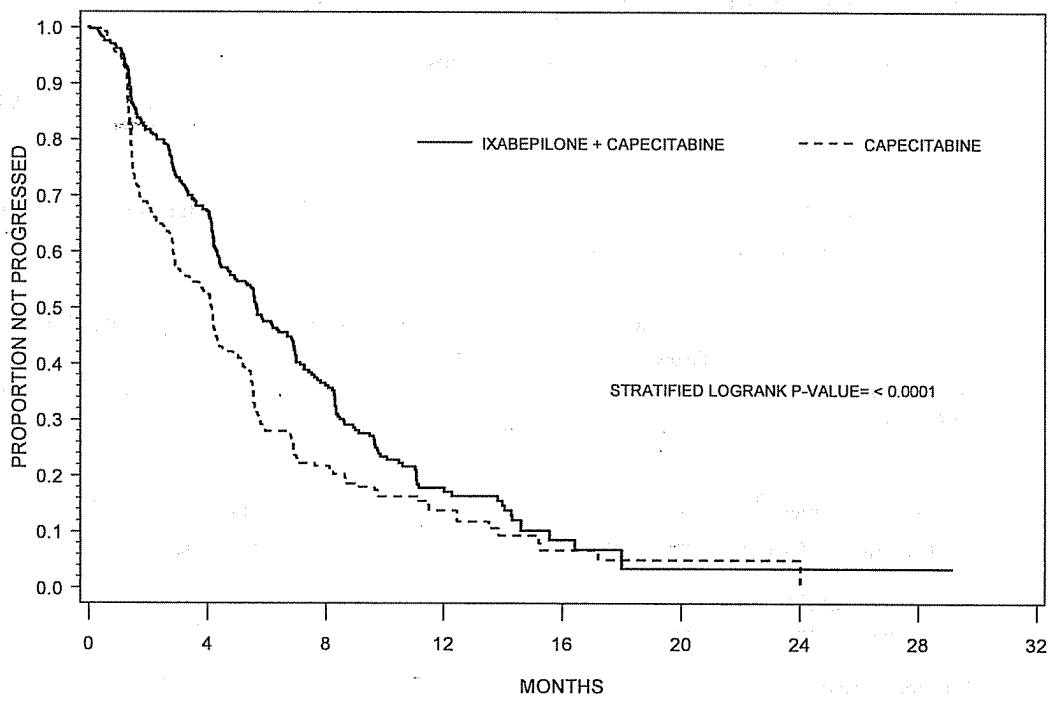
a Patients were censored for PFS at the last date of tumor assessment prior to the start of subsequent therapy. In patients where independent review was not available PFS was censored at the randomization date.

b For the hazard ratio, a value less than 1.00 favors combination treatment, CI adjusted for interim analysis.

c Stratified by visceral metastasis in liver/lung, prior chemotherapy in metastatic setting, and anthracycline resistance.

d Cochran-Mantel-Haenszel test

Figure 1: **Progression-free Survival Kaplan Meier Curves**



Monotherapy

IXEMPRA was evaluated as a single agent in a multicenter single-arm study in 126 women with metastatic or locally advanced breast cancer. The study enrolled patients whose tumors had recurred or had progressed following two or more chemotherapy regimens including an anthracycline, a taxane, and capecitabine. Patients who had received a minimum cumulative dose of 240 mg/m^2 of doxorubicin or 360 mg/m^2 of epirubicin were also eligible. Tumor progression or recurrence were prospectively defined as follows:

- Disease progression while on therapy in the metastatic setting (defined as progression while on treatment or within 8 weeks of last dose),
- Recurrence within 6 months of the last dose in the adjuvant or neoadjuvant setting (only for anthracycline and taxane),
- HER2 positive patients must also have progressed during or after discontinuation of trastuzumab.

In this study, the median age was 51 years (range, 30-78), and 79% were White, 5% Black, and 2% Asian, Karnofsky performance status was 70-100%, 88% had received two or more prior chemotherapy regimens for metastatic disease, and 86% had liver and/or lung metastases. Tumors were ER-positive in 48% of patients, ER-negative in

44%, HER2-positive in 7%, HER2-negative in 72%, and ER-negative, PR-negative, HER2-negative in 33%.

IXEMPRA was administered at a dose of 40 mg/m^2 intravenously over 3 hours every 3 weeks. Patients received a median of 4 cycles (range 1 to 18) of IXEMPRA therapy.

Objective tumor response was determined by independent radiologic and investigator review using RECIST. Efficacy results are presented in Table 8.

Table 8: Efficacy of IXEMPRA in Metastatic and Locally Advanced Breast Cancer

Endpoint	Result
Objective tumor response rate (95% CI)	
IRR Assessment [a] (n = 113)	12.4% (6.9 - 19.9)
Investigator Assessment (n = 126)	18.3% (11.9 - 26.1)
Time to response [b] (n = 14)	
Median, weeks (min - max)	6.1 (5 - 54.4)
Duration of response [b] (n = 14)	
Median, months (95% CI)	6.0 (5.0 - 7.6)

a All responses were partial.

b As assessed by IRR.

15 REFERENCES

1. Preventing Occupational Exposures to Antineoplastic and Other Hazardous Drugs in Health Care Settings. NIOSH Alert 2004-165.

2. OSHA Technical Manual, TED 1-0.15A, Section VI: Chapter 2. Controlling Occupational Exposure to Hazardous Drugs. OSHA, 1999. http://www.osha.gov/dts/osta/otm/otm_vi/otm_vi_2.html

3. American Society of Health-System Pharmacists. ASHP guidelines on handling hazardous drugs. *Am J Health-Syst Pharm.* 2006;63:1172-1193.

4. Polovich, M., White, J. M., & Kelleher, L.O. (eds.) 2005. Chemotherapy and biotherapy guidelines and recommendations for practice (2nd. ed.) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

IXEMPRA is supplied as a *Kit* containing one vial of IXEMPRA™ (ixabepilone) for injection and one vial of DILUENT for IXEMPRA.

NDC 0015-1910-12	IXEMPRA™ *Kit* containing one vial of IXEMPRA™ (ixabepilone) for injection, 15 mg and one vial of DILUENT for IXEMPRA, 8 mL
NDC 0015-1911-13	IXEMPRA™ *Kit* containing one vial of IXEMPRA™ (ixabepilone) for injection, 45 mg and one vial of DILUENT for IXEMPRA, 23.5 mL

IXEMPRA *Kit* must be stored in a refrigerator at 2° C to 8° C (36° F to 46° F). Retain in original package until time of use to protect from light.

Procedures for proper handling and disposal of antineoplastic drugs [see *References (15)*] should be followed. To minimize the risk of dermal exposure, impervious gloves should be worn when handling vials containing TRADENAME, regardless of the setting, including unpacking and inspection, transport within a facility, and dose preparation and administration.

17 PATIENT COUNSELING INFORMATION

See FDA-Approved Patient Labeling (17.6)

17.1 Peripheral Neuropathy

Patients should be advised to report to their physician any numbness and tingling of the hands or feet [see *Warnings and Precautions (5.1)*].

17.2 Fever/Neutropenia

Patients should be instructed to call their physician if a fever of 100.5° F or greater or other evidence of potential infection such as chills, cough, or burning or pain on urination develops [see *Warnings and Precautions (5.2)*].

17.3 Hypersensitivity Reactions

Patients should be advised to call their physician if they experience urticaria, pruritus, rash, flushing, swelling, dyspnea, chest tightness or other hypersensitivity related symptoms following an infusion of IXEMPRA [see *Warnings and Precautions (5.4)*].

17.4 Pregnancy

Patients should be advised to use effective contraceptive measures to prevent pregnancy and to avoid nursing during treatment with IXEMPRA [see *Warnings and Precautions (5.5)* and *Use in Specific Populations (8.1, 8.3)*].

17.5 Cardiac Adverse Reactions

Patients should be advised to report to their physician chest pain, difficulty breathing, palpitations or unusual weight gain [see *Warnings and Precautions (5.6)*].

17.6 FDA-Approved Patient Labeling

Patient Information
IXEMPRA™ *Kit* (pronounced as ĭk-'sĕm-pră)
(ixabepilone)

for Injection, for intravenous infusion only

Read the Patient Information that comes with IXEMPRA before you start receiving it and before each injection. There may be new information. This leaflet does not take the place of talking with your healthcare provider about your medical condition or your treatment.

What is the most important information I should know about IXEMPRA?

Your healthcare provider should do blood tests to check your liver function:

- before you begin receiving IXEMPRA
- as needed while you are receiving IXEMPRA

If blood tests show that you have liver problems, do not receive injections of IXEMPRA along with the medicine capecitabine. Taking these two medicines together if you have liver problems increases your chance of serious problems. These include: serious infection and death due to a very low white blood cell count (neutropenia).

What is IXEMPRA?

IXEMPRA is a cancer medicine. IXEMPRA is used alone or with another cancer medicine called capecitabine. IXEMPRA is used to treat breast cancer, when certain other medicines have not worked or no longer work.

Who should not take IXEMPRA?

Do not receive injections of IXEMPRA if you:

- are allergic to a medicine, such as TAXOL®, that contains Cremophor® EL or polyoxyethylated castor oil
- have low white blood cell or platelet counts. Your healthcare provider will check your blood counts.
- are also taking a cancer medicine called capecitabine and you have liver problems. **See "What is the most important information I should know about IXEMPRA?"**

What should I tell my healthcare provider before receiving IXEMPRA?

IXEMPRA may not be right for you. Before you receive IXEMPRA, tell your healthcare provider about all of your medical conditions, including if you:

- have liver problems
- have heart problems or a history of heart problems
- have had an allergic reaction to IXEMPRA. You will receive medicines before each injection of IXEMPRA to decrease the chance of an allergic reaction. See "How will I receive IXEMPRA?"
- are pregnant or plan to become pregnant. You should not receive IXEMPRA during pregnancy because it may harm your unborn baby. Talk with your healthcare provider about how to prevent pregnancy while receiving IXEMPRA. Tell your healthcare provider right away if you become pregnant or think you are pregnant while receiving IXEMPRA.
- are breast-feeding. It is not known if IXEMPRA passes into breast milk. You and your healthcare provider should decide if you will take IXEMPRA or breast feed. You should not do both.

Tell your healthcare provider about all the medicines you take, including prescription and non-prescription medicines, vitamins and herbal supplements.

IXEMPRA and certain other medicines may affect each other causing side effects. IXEMPRA may affect the way other medicines work, and other medicines may affect how IXEMPRA works. Know the medicines you take. Keep a list of your medicines with you to show your healthcare provider.

How will I receive IXEMPRA?

IXEMPRA is given by an injection directly into your vein (intravenous infusion). IXEMPRA is usually given once every three weeks. Each treatment with IXEMPRA will take about 3 hours.

Your healthcare provider will decide how much IXEMPRA you will receive and how often you will receive it.

To lower the chance of allergic reaction, you will receive other medicines about 1 hour before each treatment with IXEMPRA. (See "What are the possible side effects of IXEMPRA?")

If you have an allergic reaction to IXEMPRA, you will receive a steroid medicine before future doses of IXEMPRA. You may also need to receive your doses of IXEMPRA more slowly.

What should I avoid while receiving IXEMPRA?

IXEMPRA contains alcohol. If you are dizzy or drowsy, avoid activities that may be dangerous, such as driving or operating machinery.

Do not drink grapefruit juice while receiving IXEMPRA. Drinking grapefruit juice may cause you to have too much IXEMPRA in your blood and lead to side effects.

What are the possible side effects of IXEMPRA?

IXEMPRA may cause serious side effects including:

- **Numbness, tingling, or burning in the hands or feet can occur while taking IXEMPRA (neuropathy).** These symptoms may be new or get worse while you are receiving IXEMPRA. These symptoms often occur early during treatment with IXEMPRA. Tell your healthcare provider if you have any of these symptoms. Your dose of IXEMPRA may need to be decreased, stopped until your symptoms get better, or totally stopped.
- **Low white blood cell count (neutropenia).** White blood cells help protect the body from infections caused by bacteria. If you get a fever or infection when your white blood cells are very low, you can become seriously ill and die. You may need treatment in the hospital with antibiotic medicines. Your healthcare provider will monitor your white blood cell count often with blood tests. Tell your healthcare provider right away or go to the nearest hospital emergency room if you have a fever (temperature above 100.5° F) or other sign of infection, such as chills, cough, burning or pain when you urinate, any time between treatments with IXEMPRA.
- **Allergic Reactions.** Severe allergic reactions can occur with IXEMPRA and may cause death in rare cases. Allergic reactions are most likely to occur while IXEMPRA is being injected into your vein. Tell your healthcare provider right away if you get any of the following signs and symptoms of an allergic reaction:
  - itching, hives (raised itchy welts), rash.
  - flushed face

- sudden swelling of face, throat or tongue
- chest tightness, trouble breathing
- feel dizzy or faint
- feel your heart beating (palpitations)
- **Harm to an unborn child.** See "What should I tell my healthcare provider before taking IXEMPRA?"
- **Heart problems.** IXEMPRA might cause decreased blood flow to the heart, problems with heart function, and abnormal heart beat. This is seen more often in patients who also take capecitabine. **Tell your healthcare provider right away if you have any of the following symptoms:**
- chest pain,
- difficulty breathing,
- feel your heart beating (palpitations), or
- unusual weight gain.

The most common side effects with IXEMPRA used alone or with capecitabine may include:

- tiredness
- loss of appetite
- disorders of toenails and fingernails
- hair loss
- fever
- decreased red blood cells (anemia)
- joint and muscle pain
- headache
- decreased platelets (thrombocytopenia)
- nausea, vomiting, diarrhea, constipation, and abdominal pain
- sores on the lip, in the mouth and esophagus
- tender, red palms and soles of feet (hand-foot syndrome) that looks like a sunburn; the skin may become dry and peel. There may also be numbness and tingling.

Tell your healthcare provider about any side effect that bothers you or that does not go away.

These are not all of the side effects of IXEMPRA. Ask your healthcare provider or pharmacist for more information if you have questions or concerns.

General information about IXEMPRA?

This patient information leaflet summarizes the most important information about IXEMPRA. Medicines are sometimes prescribed for purposes other than those listed in a Patient Information Leaflet. If you would like more information about IXEMPRA, talk with your healthcare provider. You can ask your healthcare provider or pharmacist for information about IXEMPRA that is written for health professionals. For more information about IXEMPRA, call 1-800-721-5072.

IXEMPRA™ (ixabepilone) for injection Manufactured by: Baxter Oncology GmbH, 33790 Halle/Westfalen, Germany

DILUENT for IXEMPRA Manufactured by: Baxter Oncology GmbH, 33790 Halle/Westfalen, Germany

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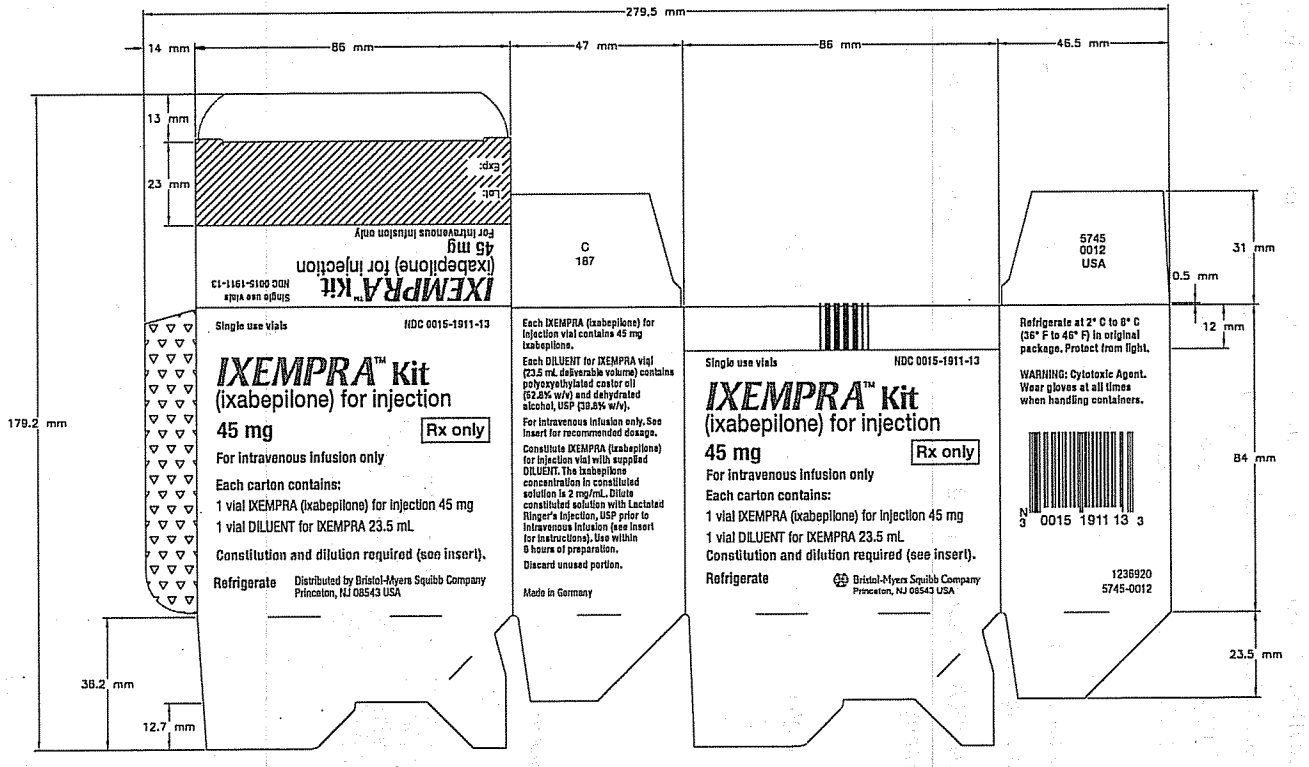

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